The Role of TGF-β1 Signalling in Peripheral $CD4^+$ T Cells

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DISCLAIMER

The thesis is based upon and partly adapted from the publication:

Inducible $CD4^+$ T cell-specific ablation of TGF-β signalling reveals a role in homeostasis but not in tolerance and T_{reg} cell function.

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CONTENTS

SUMMARY

Transforming growth factor-β1 (TGF-β1) is a cytokine with pleiotropic functions within the immune system. Roles of TGF-β are the control of development, proliferation, survival, maintenance and differentiation of $CD4^+$ T lymphocytes. TGF-β signalling is strongly implicated in peripheral tolerance and maintenance of regulatory T (T_{reg}) cells. TGF-β was also reported to inhibit the terminal differentiation of $CD4^+$ T cells into Th1 and Th2 subsets and it initiates Th17 cell polarization in a context-dependent manner. Constitutive abrogation of TGF-β signalling in T cells has been reported to result in a rapidly lethal autoimmunity.

To study the specific role of TGF-β receptor II (TR2) in peripheral $CD4^+$ T cells we generated CD4-$CreER^{t2}$ mice and inducibly removed the receptor. The ablation of the TR2 was specific to $CD4^+$ T cells leaving $CD8^+$ T cells unaffected. Unexpectedly, induced deficiency of TR2 in peripheral $CD4^+$ T cells did not result in autoimmunity or impaired tolerance. No signs of autoimmune inflammation were observed in tamiCD4TR2 mice even after thymectomy and long term tamoxifen treatment. T_{reg} cells in our model retained their suppressive abilities *in vitro* and *in vivo*. Both, effector memory and Treg cells showed hyperproliferation while naive T cell numbers decreased through apoptosis. In contrast to previous reports no changes in NK and NKT cell compartment were observed in our model. In addition, the ablation of TGF-β signalling in peripheral $CD4^+$ T cell did not lead to their spontaneous differentiation into Th1 or Th2 subsets.

We conclude that TGF-β signalling in peripheral $CD4^+$ T cells does not play a critical role in tolerance but acts as a negative regulator of T_{reg} and effector memory T cell homeostasis.

ZUSAMMENFASSUNG

Der Transforming growth factor-β1 (TGF-β1) ist ein Zytokin, das im Immunsystem vielfältige Funktionen ausübt. Er spielt eine entscheidende Rolle in der Kontrolle der Entwicklung, Proliferation, Erhaltung, Differenzierung und dem Überleben von $CD4^+$ T-Lymphozyten. Der TGF-β Signalweg wurde mit der Erhaltung der peripheren Immuntoleranz sowie regulatorischen T-Zellen in Verbindung gebracht. Zudem wurde gezeigt, dass die Differenzierung in Th1 und Th2-Zellen von TGF-β gehemmt wird, während er die Th17-Entwicklung kontextabhängig induzieren kann. Nach der konstitutiven Inaktivierung des TGF-β Signalweges in T-Zellen in Mäusen haben die Tiere eine rasch tödlich verlaufende Autoimmunkrankheit entwickelt.

Um die Rolle des TGF-β Rezeptor II (TR2) spezifisch in peripheren $CD4^+$ T-Zellen zu untersuchen, haben wir CD4-$CreER^{t2}$-Mäuse entwickelt, die es erlauben, den Rezeptor induziert zu entfernen. Die TR2-Ablation fand in diesem neuen System spezifisch in $CD4^+$ T-Zellen statt, wobei $CD8^+$ T-Zellen unbeeinflusst blieben. Nach der Entfernung des TR2 in peripheren $CD4^+$ T-Zellen konnten wir unerwarteterweise keine Entwicklung von Autoimmunität oder eine andersweitig beeinträchtigte Immuntoleranz feststellen. Auch nach Thymektomie und einer Langzeitbehandlung mit Tamoxifen zeigten die Mäuse keine Anzeichen von autoimmunen Entzündungsprozessen. Regulatorische T-Zellen blieben in ihren immunsuppressiven Eigenschaften nach der Unterbrechung des TGF-β Signalweges *in vitro* und *in vivo* unverändert. T_{reg}-Zellen und Effektor-Gedächtnis-T-Zellen zeigten eine Hyperproliferation, die Anzahl naiver T-Zellen hingegen nahm durch Apoptose ab. Im Gegensatz zu vorherigen Studien wurden keine Veränderungen in NK und NK T-Zellen festgestellt. Die Entfernung des TR2 in $CD4^+$ T-Zellen hat auch nicht zu einer spontanen Differenzierung von naiven in Th1 oder Th2-Zellen geführt.

Diese Ergebnisse lassen darauf schliessen, dass der TGF-β Signalweg in peripheren $CD4^+$ T-Zellen keine entscheidende Rolle in der Erhaltung von Immuntoleranz spielt, sondern die Homöostase von regulatorischen und Effektor-Gedächtnis T-Zellen negativ reguliert.

INTRODUCTION

Transforming growth factor beta 1 (TGF-β1)

The transforming growth factor-β (TGF-β) family consists of a large number of structurally and functionally related proteins involved in many biological processes during development, lineage commitment, proliferation, migration and survival of cells. The TGF-β family contains two subfamilies of cytokines defined by sequence similarity and the specific signalling pathways that they activate.

The TGF-β subfamily comprises three homologous isoforms in mammals: TGF-β1, TGF-β2 and TGF-β3. They play a major role in control of cell cycle, proliferation and differentiation, as well as in immunosuppression, wound healing and production of extracellular matrix [1]. The three isoforms are encoded by three different genes and exert different functions. TGF-β1 is the predominant form expressed by immune cells in mammals and crucial for the regulation of immune responses. In humans its major functions in angiogenesis and cancerogenesis have also been described [2-3]. The most crucial for immune system is TGF-β1, the other two isoforms were so far not found to play a significant role [2, 4].

Synthesis and signaling pathways

TGF-β is synthesized as a precursor (pre-pro-protein) that is cleaved before secretion. The resulting TGF-β propeptide consists of the mature homodimer TGF-β and the non-covalently associated latency-associated peptide (LAP) [5-6]. This latent form can be either secreted directly or after binding the latent-TGF-β-binding-protein (LTBP), which play important role in targeting the TGF-β to the extracellular matrix [5]. Extracellulary matrix mature TGF-β has to be liberated from the constraints of the associated proteins in order to bind to its receptor [1]. In this process plasmin and matrix proteinases, reactive oxygen species, the protein thrombospondin-1 and integrins αvβ6 and αvβ8 are involved [5, 7-8]. Both integrins bind to

an RGD motif in LAP but they act through different mechanisms. αvβ6 mediated TGF-β1 activation occurs through the conformational changes in LAP whereas αvβ8-induced activation requires metaloproteinases. There are also studies showing that TGF-β can be attached to the cell-surface and presented to the target cells [9-11].

The active form of a TGF-β is a homodimer that is stabilized by hydrophobic interactions which are further strengthened by an intersubunit disulfide bridge [12-13]. The cytokine signals through the tetrameric complex of TGF-βRI and TGF-βRII each containing an intracellular serine/threonine kinase domain [1, 14-15].

The best elucidated signalling pathway used by TGF-β involves Smad proteins which deliver the signal into the nucleus [16-17]. By activation of TGF-βRI, the receptor is able to phosphorylate receptor-regulated Smads (R-Smad): Smad2 and Smad3. Phosphorylated R-Smads form homotrimers and interact with Co-Smad4. Smads complexes can be transported to the nucleus via a non-canonical nuclear import pathway resulting from the ability of these proteins to interact with the components of the nuclear pore [18-19]. In the nucleus Smad complexes associate with other transcription factors, as by themselves they have low DNA binding affinity. Gene regulation by Smad proteins is then mediated through the recruitment of chromatin remodelling enzymes histone-acetyl transferases (HAT) e.g. CBP/p300, histone deacetylases (HDAC) e.g. Sno/Ski [20]. Also involved in TGF-β signalling also involved is a third group of Smad protein, inhibitory Smads (I-Smad). Activated Smad2/3/4 complexes upregulate the expression of inhibitory Smad7. In the cytoplasm Smad7 competes with Smad2 and 3 for binding to TGF-βRI and mediates the degradation of the receptor by E3 ubiquitinase complexes [21-22].

Studies performed with cells deficient for Co-Smad4 or with mutated TGF-βRI showed that other than Smad-mediated pathways for TGF-β signalling exist [23-24] that involve the MAP-kinase cascades Erk, JNK p38 or activation of PI3K [25]. However activation of Erk

and JNK MAPK pathways can also result in phosporylation of Smad proteins. Thus, Smad-dependent and Smad-independent pathways and the cross-talk of both determine the effect of TGF-β signalling on a cell.

In the immune system, the action of TGF-β1 probably affects most immune-cell types as a result of broad distribution of the cognate receptor on these cells. It can be also synthesize by cells belonging to both innate and adaptive immune system. TGF-β1 is also one of the most crucial cytokine in T cell biology. It affects T cell development, differentiation, proliferation and survival. The first study to show that TGF-β has a potent influence on the regulation of T cells was published by Kehrl et al. in 1986. They showed that human T cells were inhibited in IL-2 mediated proliferation by the presence of TGF-β *in vitro*. Furthermore, they noticed that activated T cells themselves were able to produce TGF-β [26]. Later studies showed the role of TGF-β in the differentiation of T cells into effector cells and its role in central and peripheral tolerance.

Acquired immunity and tolerance against self

The immune system in the healthy individuals is balanced between driven tolerance against self-antigen and pathogen-driven immunity. Functional tolerance is based on the development of central and peripheral tolerance. The term central tolerance applies to the process of deletion of autoreactive lymphocytes during maturation in the central lymphoid organs, while peripheral tolerance applies to functional suppression in the periphery of autoreactive lymphocytes that have escaped elimination.

Thymic development of the T cell and induction of central tolerance

T cells are key players of adaptive immune system. The repertoire of different clones is able to respond to a seemingly limitless number of potential foreign antigens while simultaneously not responding to self-antigens expressed in the various tissues.

T cells develop from progenitors derived from pluripotent hematopoetic stem cells in the bone marrow [27] and migrate through the blood to the thymus where they mature. Developing T cells pass in the thymus through a series of distinct phases that are marked by the status of T-cell receptor genes, expression of the T cell receptor (TCR) and by changes of the expression of cell surface proteins. Progenitors that enter to the thymus from the bone marrow are called double-negative (DN) because the co-receptors CD4 and CD8 are not expressed on their surface. In this stage thymocytes lack most of the markers characteristic for mature T cells and the TCR genes are not rearranged. Those cells give rise to either a minor population of γ:δ or to a major of α:β T cells, only the latter will be in the focus of this thesis [28-29]. (**Fig.I**)

Developing thymocytes undergo stringent selection processes that shape the T cell repertoire. The first checkpoint, called beta-selection ensures that only those DN thymocytes that have successfully rearranged their TCRβ locus progress to the $CD4^+CD8^+$ double positive stage (DP). Beta-selection is dependent on the signalling from the pre-T-cell receptor which consist of the β-chain and a surrogate pre-T-cell receptor α chain called pTα (for rev: [30-31]). (**Fig.I**)

After progression to the DP stage and rearrangement of the TCRα locus developing T cells interact with thymic stromal cells presenting peptides in the context of MHC. These interactions are necessary to shape the repertoire of T cells via processes called positive and negative selection. (for rev: [32-33]). It is still not completely understood how one receptor can drive opposite cellular response: differentiation, proliferation or apoptosis. The most recent publications showed that quantifiable variations in the affinity of a T cell receptor for a peptide–MHC complex can result in qualitatively different signals and thus determine thymocyte fate [34-35]. Failure of TCR to interact with self MHC-ligand complex at the appropriate level of affinity results in so-called death by neglect of the respective thymocytes. During this process an estimated 90 to 95% of thymocytes undergo apoptosis. [36].

Positive selection takes place in the cortical part of the thymus and the peptide-MHC complexes are presented by cTECs (thymic epithelial cells). These cells are able to generate MHC-bound peptides through unique pathways (for rev: [33]). Subsequent to positive selection and CD4 or CD8 lineage commitment thymocytes move to the medulla where they stay for four to five days [37]. During this period thymocytes interact with medullary APCs: thymic epithelial cells (mTEC) and dendritic cells (DCs) that present self-peptide in the context of MHC. T cells that recognize these peptides with an affinity that is too high undergo apoptosis, a process that is called negative selection and plays a key role in central tolerance. The ectopic expression of tissue-restricted antigens in the thymus is controlled in part by the autoimmune regulator (AIRE) gene [32, 38-39]. Targeted disruption of AIRE in the mouse leads to autoimmune inflammation in many organs [40]. A similar observation is made in human patients where the mutation in AIRE leads to development of autoimmune polyendrocrinopathy candidiasis ectodermal dystrophy (APECED) [41]. Numerous experiments showed that any disruption of mTEC development or medullary architecture result in development of systemic autoimmunity, which emphasize the role of medulla in induction of central tolerance.

T cells that survived both processes, positive and negative selection differentiate into single positive $CD4^+$ or $CD8^+$ T cells depending on their respective abilities to bind invariant sites on MHCII and MHCI molecules and leave to the periphery as naïve T cells.

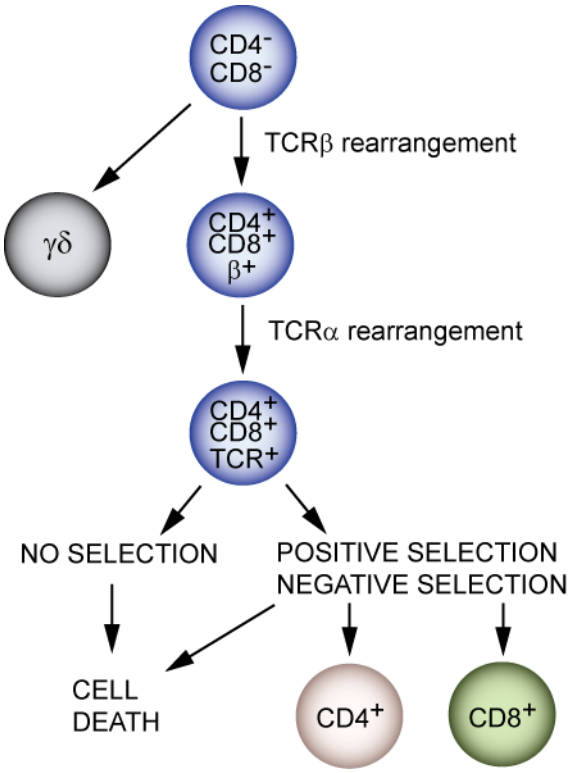

Fig. I Thymic T cell development

T cells progenitor enters the thymus near the cortico-medullary junction. DN thymocytes give rise to minor population of γδ T cells and a major population of αβ T cells. Thymocytes that successfully rearrange the β locus progress to the DP stage. After rearrangement of the α locus DP thymocytes interact with self-MHC ligand complex. Failure of TCR to interact with such complexes on the certain level of affinity results in cell death. DP thymocytes undergo positive and negative selection which can result in apoptosis or development of CD4 and CD8 single positive T cells.

TGF-β signalling was shown to play a role in the thymic development of different T cell subsets. Early studies, performed *in vitro,* showed that TGF-β was able to inhibit the proliferation of human thymocytes in response to IL-2 or IL-7 [42]. On the basis of another *in vitro* analysis a role of TGF-β in the development of $CD8^+$ T cells was proposed because this cytokine promoted CD8 expression in $CD3^-$ thymocytes [43]. Studies performed on fetal thymic organ cultures gave similar results [44]. TGF-β1 was shown to be expressed in murine cortical epithelial cells [44] as well as human epithelial cells where TGF-β regulates cytokine

expression by these cells directly affecting T cell development [45]. It was suggested that TGF-β1 produced by TEC inhibits the progression of DN thymocytes to the DP stage [44]. Experiments performed with TGF-β1 deficient mice or conditional knockout of TGF-β in immune cells allowed to confirm *in vivo* the observations based on *in vitro* studies. In the TGF-β1 knockout strain which develop highly aggressive autoimmune disease total thymus cellularity was decreased. At the same time hyperplasia of the medulla and reduction of the thymic cortex were reported [46]. Later studies with the same model showed reduction in the DP thymocytes and increase in percentage of DN and CD4 SP compartments [47]. Studies using more specific mouse model that do not have TGF-β receptor on cells of hematopoietic origin or on T cells resulted in partially contradictory observations. In TGF-βRII deficiency in hematopoietic cells (by use of Mx1-cre) no aberrant thymic T cell development was observed, only $CD8^+$ SP thymocytes showed increased proliferation [48]. In contrast, deletion of TGF-βRII in T cells (CD4 promoter) in one of the studies resulted in decreased $TCR^{hi}CD8^+$ SP T cells compartment and accumulation of DP thymocytes [49]. The studies using this model revealed an indispensable role of TGF-β signaling in the thymic differentiation of NKT cells [49-50]. Furthermore, it was shown that TGF-β directly orchestrate iNKT cell development directly through Smad 4, Tif-1g and the Tif-1g-Smad4 independent pathways [51].

Peripheral tolerance

Despite stringent selection in the thymus even a healthy individual immune system contains self-reactive T cells that have escaped central tolerance. Thus, development of autoimmune response must be prevented by additional, peripheral mechanisms.

Tolerogenic DCs are one of the primary mediators of peripheral tolerance [52-53]. They resemble immature DCs whereby the common features are the low expression of MHCII, CD40 and CD80/86 due to the absence of inflammatory molecules and “danger signals” [54]. The recognition of tissue-restricted antigen on tolerogenic mature DCs by T cells leads to

their functional inactivation and/or peripheral deletion in secondary lymphoid organs. DCs were also shown to contribute to the development of induced T_{reg} cells (iT_{reg}) and regulatory T cell that produce IL-10 in high amounts (Tr1)[55].

The other components of peripheral tolerance are regulatory T cells. Those cells have the potential to suppress self-reactive lymphocytes that recognize antigens different from those recognized by T_{reg} cells, a phenomenon called regulatory tolerance or dominant suppression. Absence or dysfunction of regulatory T cells lead to development of aggressive autoimmune diseases [56-57]. The protective role of regulatory T cells was shown in several autoimmune disease in mice like inflammatory bowel disease (IBD), SLE or experimental autoimmune encephalomyelitis (EAE) the mouse model for multiple sclerosis [58].

Regulatory T cells

Regulatory T cells are a heterogeneous subpopulation of $CD4^+$ T cells with different developmental origin which negatively control almost every adaptive immune response, either physiological or pathological. In 1995 a subset of $CD4^+$ T cells with high expression of IL-2Rα (CD25) was found to have suppressive functions [59]. The main fraction of this cell type is called nT_{reg} which is of thymic origin. However, naïve T cells in the periphery were also shown to be able to convert into cells with regulatory function. (**Fig.II**). These Foxp3+ regulatory T cells of peripheral origin are now termed induced or adaptive T_{reg} (iT_{reg}). It was reported that naïve T cells upon TCR and TGF-β stimulation induce expression of Foxp3 and acquire suppressive functions [60]. The mechanism by which TGF-β induces transcription of Foxp3 involves cooperation of the transcription factors STAT3 and NFAT at a Foxp3 gene enhancer element [61-62]. TGF-β induction of Foxp3 expression was shown to be mediated by the recruitment of its downstream transcription factor Smad3 to a Foxp3 enhancer element [63]. TGF-β-induced Foxp3 expression is augmented in the presence of IL-2 which activates transcription factor STAT5 [64-65].

Other possible ways to generate these cells *in vivo*, including treatment of the mice with very little dose of antigens, has been described by several groups [66]. iT_{reg} are especially present in gut and mesenteric LN, where $CD103^+$ DCs are the key cell type involved in their induction [67]. GALT DCs have the ability to increase the gut homing receptor α4β7 integrin on T cells and additionally produce high amounts of retinal dehydrogenase which was shown to enhance iT_{reg} generation [55, 68-69]. It is not clear which DC subset except DECT 205^+ is responsible for the iT_{reg} generation in other lymphoid organs. Apart from the dendritic cells, TGF-β was also shown to play a the role in iT_{reg} development *in vivo*. Abrogation of TGF-β signalling in naïve T cells resulted in their decreased ability to convert into iT_{reg} upon stimulation of low dose of antigen [70].

So-called Tr1 and Th3 regulatory T cells that do not express Foxp3 can also be induced in the periphery of naïve T cell. Tr1 cells can develop from $CD4^+$ T cells after antigenic stimulation in the presence of IL-10, and they become IL-10 and TGF-β producers [71-72]. Antigen-specific TGF-β producing Th3 cells were originally obtained from mice that were tolerant to orally administrated antigen. (for rev: [73]). Some recent studies show that Th3 express Foxp3 as well [74](**Fig. II).**

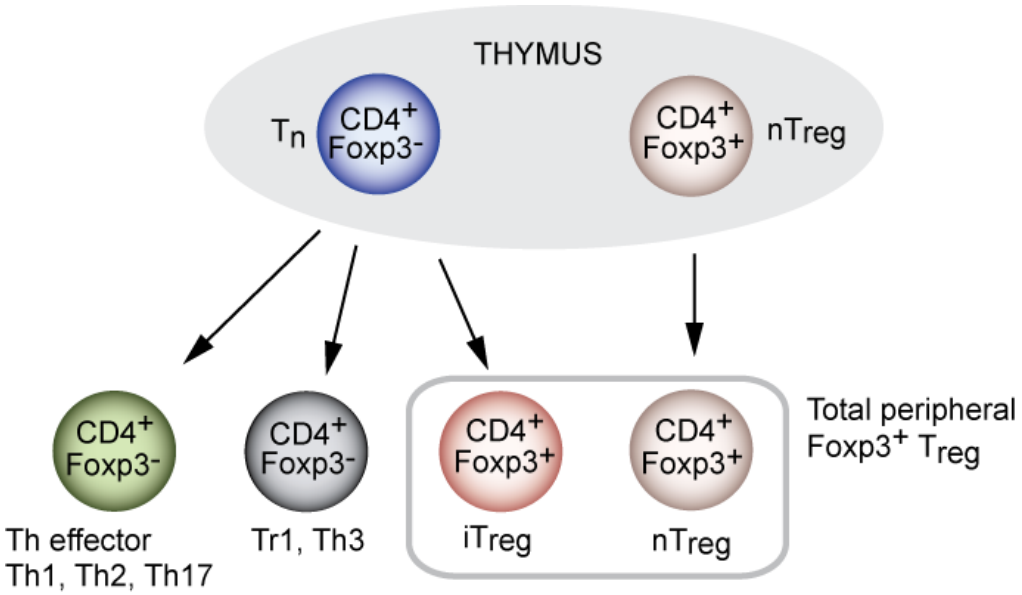

Fig.II Thymic and peripheral generation of regulatory T cells

The peripheral pool of regulatory T cells comprises Foxp3+ nT_{reg} of thymic origin and iT_{reg} generated in the periphery as well as $Foxp3^-$ regulatory T cells (Tr1, Th3).

Mechanisms of Treg-mediated suppression

$Foxp3^+$ regulatory T cells were shown to act through several distinct mechanisms on both T cells and antigen presenting cells (for rev:[75-76]). *In vitro* studies revealed a broad spectrum of molecules and processes that contribute to T_{reg} suppressive activities. Yet, not all of them were shown to play a role *in vivo*. Regulatory T cells are able to inhibit T cell proliferation, activation and differentiation but they seem to also target APCs [77-78]. T_{reg} cells were shown to mediate suppression of T cells by inhibiting the induction of IL-2 mRNA [79-81] or by competition of IL-2 consumption [82]. Regulatory T cells may also act on responding T cells through soluble factors like IL-10, TGF-β or IL-35. Regulatory T cells that were not able to produce IL-35 were shown to be inefficient in IBD curing [83]. One other potential mechanism for T_{reg}-mediated suppression of responder T cell is granzyme-mediated cytolysis of target cells [84]. Galectin-1, a member of a highly conserved family of β-galactosidase

binding proteins, was also reported to be highly expressed on T_{reg} and can interact with receptors on effector T cells, which results in cell cycle arrest or apoptosis [85].

One of the major functions of regulatory T cells is the inhibition of priming and differentiation of effector T cells. That is why antigen presenting cells are also the target for Treg cells. Among many mechanisms which are used by Treg cells to regulate APC function the CTLA-4 mediated suppression is considered to be the most crucial one. It has been proposed that the interaction of CTLA-4 on T_{regs} with CD80 and CD86 expressed by DC limits the capacity of these cells to stimulate naïve T cells through CD28. It was shown that the absence of CTLA-4 on T_{reg} cells leads to the development of systemic autoimmune disease, which confirms the importance of CTLA-4 for proper T_{reg} function [86]. A number of other mechanisms were proposed to be used by T_{regs} to decrease the co-stimulatory function of DCs. Among them, catalytic inactivation of extracellular ATP by CD39 expressed on T_{regs} was shown to be one of the most important one [87]. It was also shown that T_{reg} cells can suppress DCs by strong binding of LAG-3 (CD223), a CD4 homolog to MHC-II expressed on dendritic cells, which leads to inhibition of their maturation and decrease of their co-stimulatory functions [88].

The role of TGF-β for the T_{reg} function is still not clear and data coming from *in vitro* and *in vivo* experiments are sometimes contradictive. In 2001 Nakamura and colleagues proposed the hypothesis that regulatory T cells carry bound TGF-β on their surface. This membrane-bound TGF-β can be directly presented to target cells which would than locally convert it to the active form [89]. In their studies, blocking of TGF-β signalling diminished T_{reg}-mediated suppression. Similar studies performed by the group of Piccirillo [90] gave the opposite results. Up to now most of the studies performed with human and mouse T_{reg} cells with anti-TGF-β antibodies *in vitro* failed to demonstrate decrease suppressive activity of regulatory T cells. More recent studies showed that T_{reg} cells after strong TCR stimulation show on the

surface latency associated peptide (LAP) and very likely the latent form of TGF-β [91-92]. The observations coming from the *in vivo* studies also do not give the clear answer how TGF-β contributes to the suppressive capacity of T_{reg} cells. Transfer of TGF-β1-/- splenocytes to Rag2-/- animals cause a disease that was exacerbated after depletion of TGF-β1-/- T_{reg} cells suggesting that TGF-β is not essential for T_{reg} suppression *in vivo* [93]. However, the regulatory T cells from TGF-β1-/- or expressing dominant negative form of TR2 were not capable of inhibiting colitis induced by naïve T cell transfer [94]. Similar results were obtained in studies using the model with T cell specific deletion of TGF-β1. The authors found that TGF-β1-deficient T_{reg} cells were defective in inhibiting colitis induced by transferred naïve T cells, and this defect was associated with the failure to inhibit naïve T cell differentiation into Th1 lineage [95].

Generation and homeostasis of natural regulatory T cells

The term natural regulatory T cells (nTr_{eg}) applies nowadays to the $CD4^+CD25^+Foxp3^+$ T cells that in mice constitute 5 to 10 % of total $CD4^+$ T cells and represent a separate thymus-derived lineage [96]. The transcription factor Foxp3 was shown to be essential for T_{reg} development and function [97-99]. Nevertheless, recent studies rather suggest that Foxp3 is necessary to stabilize T_{reg} function and their phenotype but that does not determine the cell fate decision that turns developing thymocytes into T_{reg} cells [100-101]. However, the thymic development of regulatory T cells is still not completely understood. It is not clear which signal triggers the Foxp3 expression in the subpopulation of developing thymocytes (for rev: [102]) It has been suggested that for development of Treg cells a strong interaction between TCR and peptide-MHC complex is necessary [103]. Others showed that T_{reg} are more resistant to apoptosis. This can be partially due to the high expression of GITR [104]. Co-stimulatory molecules, especially CD28 and CD80/86, play an important role in Treg cell development [105]. One of the main features of T_{regs} is the high expression of IL-2 receptor

(CD25). The IL-2 signalling is important for survival and maintenance of regulatory T cells in medulla but its role in the generation of this CD4 T cell subset is dispensable [106]. This could be partially explained by compensatory IL-15 signalling since T_{reg} cells also express also high level of IL-2Rβ (CD122).

The role of TGF-β signaling in development of induced T_{reg} cells is well established wherease its involvement in the generation of natural T_{regs} remains controversial. The analysis of very young heterozygous TGF-β1-/- mice did not reveal differences in Foxp3+ thymocytes in comparison to control littermates [107]. Experiments performed with T cell-specific TR2-/- mouse model did not show affected development of natural regulatory T cells in the DP compartment prior to onsett of autoimmune disease in these mice [49-50]. Although in one of the studies [49] an increased population of Foxp3+ thymocytes in 2-week-old mice was reported. The latest reports using this mouse model showed increased thymic apoptosis of nT_{regs} which correlated with down-regulated expression of anti-apoptotic protein Bcl-2 and increased expression of Bim [108]. The analysis of a mouse model with T cell-specific deletion of TGF-βRI at different ages showed that thymic development of regulatory T cells was blocked in three to five day old mice but in mice older than one week lack of TGF-β signalling triggered their expansion [109]. Yet, the molecular mechanism by which TGF-β signalling regulates early nT_{reg} development is still unknown.

TGF-β is not only involved in the generation of regulatory T cells but is also thought to regulate their homeostasis and maintenance in the periphery. In secondary lymphoid organs in T cell-specific TR2-deficient mice the proportion of $Foxp3^+$ T cells is decreasing over time. The BrdU labelling however showed their increased proliferation [49-50]. Also regulatory T cells from TR1 knockout mouse fail to survive in the periphery. In a study performed with DN-TR2 model the expansion of $CD25^+$ CD4 T cells was shown, but without analysis of Foxp3 expression it remains unclear whether these cells were T_{reg} or activated conventional T

cells [110]. In another report it was shown that T cell-produced TGF-β1 is dispensable for the maintenance of T_{reg} cells [95]. These findings suggest that T_{reg} cells maintenance probably depends on TGF-β1 produced by other cells than T cells. TGF-β is also thought to regulate Foxp3 expression since the regulatory T cells that survived in the periphery of TGF-β1 null mice had diminished expression of Foxp3 [107].

Autoimmune inflammation

Autoimmune diseases occur when immune cells initiate an attack against the body's own tissues and organs. Under certain conditions, which very often remain unclear, the loss of peripheral tolerance to self-antigens leads to the expansion of self-reactive effector lymphocytes and development of inflammation in the tissues. Autoimmune diseases are usually classified into two categories: systemic and organ–specific. Systemic autoimmune diseases affect a variety of organs and usually involve a major humoral component. Systemic lupus erythematosus (SLE) and primary Sjörgen`s syndrome belong to this group. In tissue-specific autoimmune diseases only one organ is targeted. Examples of this disease type are type 1 diabetes mellitus which is caused by immune attack on insulin-pancreatic β cells [111] or multiple sclerosis (MS) in which an immune response is directed against CNS antigens [112]. In these diseases cell-mediated autoimmunity is thought to play the major role. In addition to Th cells APCs are key players in the initiation phase and during progression of autoimmune disease; mainly by producing cytokine necessary for Th cells polarization. The cause of many autoimmune diseases is still not known but certain factors are thought to be favourable for the development of autoimmunity like chronic inflammation, infections with certain pathogens or genetic predispositions [113].

Regulatory T cells play an important role in preventing autoimmune disease development. A spontaneous mutation in mouse in the Foxp3 gene (the scurfy mutation) leads to systemic autoimmune inflammation [98]. In human a single mutation in this gene results in X-linked

recessive autoimmune syndrome IPEX (immune dysregulation, polyendocrinopathy, enteropathy X-linked disease) [114]. Also, polymorphism of several genes that are controlled by Foxp3 and were shown to be important for Treg function, such as CTLA-4, CD25, IL-2 are associated with increased susceptibility to autoimmune diseases including type 1 diabetes [115]. Moreover, the *in vivo* blocking of CTLA-4 was shown to exacerbate diabetes in NOD mice [116].

The role of TGF-β signaling in T cells in the development and prevention of autoimmunity

Mice deficient for TGF-β1 were found to develop an early-onset multi-organ autoimmune disease leading to death by four weeks of age [117].These mice suffer from severe myocarditis, vasculits, hepatitis and lymphadenopathy. Deficiency of either of the two receptor subunits leads to an even more drastic phenotype with embryonic lethality between days E10.5 and E13.5 due to defects in vascularisation and hematopoesis [118-119]. Hematopoetic or T cell-specific expression of a dominant-negative receptor mutant or deficiency of either of the TGFβRs or TGF-β1 resulted in phenotypes resembling very much complete TGFβ1 deficiency with autoimmunity of varying severity. Mice with a dominant-negative form of TGF-βRII in T cells showed milder phenotype than TGF-β1-/- animals. The disease onset was delay and the lesions were mostly found in mucosal organs such as lungs and colon [110, 120]. This was probably due to incomplete abrogation of TGF-β signaling or change signaling in T cells. Mice with T cell specific deletion of TGF-βRII suffered from nearly as severe disease as TGF-β1-/- mice. They develop highly aggressive autoimmune disease that leads to death in the age of three to four [50] or five weeks [49]. Histological analysis of these mice reveled infiltrations of immune cells into multiple organs and tissues like stomach, liver, lung, pancreas and thyroid glands. The abrogation of TGF-β signaling in

T cells was also found to lead to loss of B cell tolerance since antibodies against self-antigens were detected in the sera [49]. In addition mice suffered from lymphadenopathy, diffuse hepatocellular lipidosis with lymphocytic cholangiohepatitis, and lymphocytic myositis involving both the myocardium and skeletal muscles [50]. Infiltration of leukocytes to different organs was also reported in the mouse model in which the TGF-β1 gene was disrupted in T cells [95, 121]. However, the disease onset was markedly delayed with the first syndromes appearing at the age of four month. The observed autoimmune phenotype in all of these transgenic mouse models was generally attributed to a defect in peripheral tolerance, especially in regulatory T cells and outgrowth of autoreactive T cell clones.

TGF-β was also shown to be involved in immune regulation in mouse models for human autoimmune diseases. Overexpression of TGF-β1 in MRL/lpr mice that develop systemic autoimmune disease mimicking human SLE inhibits development of this syndrome [122]. In some SLE patient decreased production of TGF-β1 was reported but it was not correlated with disease severity [123]. Systemic administration of TGF-β1 inhibits the development of collagen- induced arthritis (CIA) in mice, which is the model of rheumatoid arthritis in human [124]. Several studies on the role of TGF-β1 in type 1 diabetes mellitus were performed on NOD mice. In general overexpression of TGF-β1 in either β or α cells inhibit the disease [125-127].

Experimental autoimmune encephalomyelitis (EAE), a model for the human disease multiple sclerosis (MS) (for rev: [128]), is another example of autoimmune inflammation in which TGF-β was shown to play a role. Classical models of EAE are mainly driven by CD4+ T lymphocytes [129-131]. The disease can be divided into two phases: an initial T cell priming/activation phase and a subsequent recruitment and effector phase [132]. The effector phase involves migration of activated myelin specific T cells to the CNS, in which they cross the blood–brain barrier (BBB). Reactivated T cells in the brain initiate cascade of events,

including the secretion of chemokines that recruit predominantly macrophages to the site of inflammation.

The majority of the studies performed on the EAE model show a beneficial role of TGF-β1 in this disease. Administration of exogenous TGF-β1 to mice was able to inhibit development of the EAE [133-135]. Treatment of mice with neutralising antibodies against TGF-β enhanced the clinical severity of the disease [136]. Further beneficial effects for TGF-β were reported in the recovery phase of the EAE. Up-regulation of TGF-β along with other immunoregulatory cytokines as IL-10 and IL-4 was observed in recovering animals [137-138]. TGF-β1 is also assosiated with the induction of oral tolerance to EAE antigens because treatment of mice with low dose MBP induced supressive Th3 cells that produce high amount of TGF-β. A protective role of TGF-β on EAE development was also shown by a study with mice lacking TGF-β signalling in dendritic cells, as these animals were highly susceptible to the disease [139].

Several studies addressed the role of regulatory T cells in EAE. In general depletion of Treg cells before induction of the disease increased disease severity and mortality of the animals [140]. In the recovery phase of EAE an increased population of regulatory T cell expressing surface-bound TGF-β (in form of LAP) was reported, which suggested that remission is dependent on the immunoregulatory properties of TGF-β [9] (for rev: [141]).

In contrast, TGF-β was also described to be involved in pro-inflammatory processes, as in promoting the differentiation of Th17 cells. It was reported that animals which expressed dnTGF-βRII in T cells lacked Th17 cells and developed significantly less severe EAE [142]. An important role for action of TGF-β in priming encephalitogenic Th17 cells was suggested as addition of neutralizing TGF-β antibodies in the immunization emulsion inhibited EAE development in wild type mice. In contrast to this local administration, systemic injection of anti-TGF-β did not block Th17 differentiation and disease induction. Also, in mice deficient

for T cell-produced TGF-β1 [95] IL-17 production was strongly reduced and animals were less susceptible to the disease. In contrast, a study investigating encephalitogenicity of Th17 cells revealed that primed T cells restimulated *in vitro* in presence of TGF-β and IL-6 rather suppressed disease development whereas animals which received IL-23 driven T cells developed EAE [143]. TGF-β and IL-6 instead led to an increased expression of the immunoregulatory cytokine IL-10 by restimulated cells and thus prevented disease development.

Peripheral T cells homeostasis and subsets of $CD4^+$ T cells

Homeostasis of naïve T cells

The survival and the composition of the mature T cell pool are governed by complex homeostatic mechanisms. Naïve T cells, characterised by high expression of L-selectin (CD62L) and low expression of CD44 [144], hardly divide and their numbers remain relatively stable during the lifespan. The importance of TCR signalling for naïve T cell survival was shown by many groups. The lifespan of both $CD4^+$ and $CD8^+$ naïve T cells was shortened when they were deprived of contact with self-pMHC molecules [145-146]. Studies in which the TCR expression or TCR signalling was abrogated in mature T cells also showed a reduction in lifespan of naïve T cells [147-148]. However, these studies did not allow for concluding if the naïve T cells need the interaction with self-pMHC or if just the tonic signalling from TCR is sufficient. There are many evidences suggesting that naïve T cells have to home to secondary lymphoid organs to maintain a stable population size. Homing to the T cell zones in SLO is dependent on high expression of CD62L and CCR7 (CD197). Therefore, blocking or deletion of these molecules leads to decrease in naïve T cell lifespan [149]. The expression level of CD62L and CCR7 on naïve T cells was shown to be regulated by transcription factors Kruppel-like transcription factor KLF2 and FoxO1. The latter is also involved in regulation of IL-7rα (CD127). IL-7 together with interleukins 2, 4, 7, 9, 15 and 21

belongs to the family of γc-dependent cytokines, which use the γc chain as a receptor subunit. The main source of IL-7 in secondary lymphoid organs is a subset of fibroblastic reticular cells (FRC) that are located in T cell zones. These cells produce also the chemokines CCL19 and CCL21 which are the ligands for CCR7, thereby attracting naïve cells [149]. The survival signal induced by IL-7 binding is mediated through the activation of Jak1, Jak3 and Stat5a/b [150-151]. IL-7 and related cytokines promote cells survival by preventing the mitochondrial pathway of apoptosis [151].

In response to severe depletion of T cells naïve cells undergo spontaneous homeostatic proliferation. The process of slow lymphopenia-induced proliferation (LIP), also called homeostatic proliferation, allows the restoration of normal T cell level. Naïve T cells that undergo homeostatic proliferation acquire memory phenotype with high expression of CD44. LIP is mainly driven by interaction between TCR and pMCH and through IL-7 signalling. Nevertheless, co-stimulatory molecules such as CD24 (HSA) were also shown to play a role. Adoptive transfer of naïve T cells to the animal that chronically lymphopenic like RAG1-/- also leads to rapid homeostatic expansion that is drive by foreign antigens from commensal microflora and since it is greatly reduced in germfree environment [152].

Homeostasis of memory T cells

Memory T cells, in contrast to naïve cells, undergo intermittent cell division. Their longevity and division of both CD4 and CD8 memory T cells is independent of contact with pMHC and rely mostly on IL-7 and IL-15 [151, 153-155].

Central memory T cells express CD62L and CCR7 which allows them to enter secondary lymphoid organs. Both, $CD4^+$ and $CD8^+$ memory T cells show high expression of CD127 but CD122 is only highly expressed on $CD8^+$ memory T cells, which suggest that they are more dependent on IL-15 than $CD4^+$ T cells. Recent studies show that expression of CD122 on T cells is regulated by two T-box transcription factors: T-bet and Eomesodermin (Eomes) [156].

Unlike central memory, effector memory T cells are largely excluded from lymph nodes and instead accumulate in the spleen and in non-lymphoid peripheral organs. The homeostatic proliferation of these cells is probably mostly driven by IL-15 signalling but it is not clear whether they require interaction with pMHC (For rev: [151]).

T helper subsets and their function

$CD4^+$ T cells play a critical role during adaptive immune response. They function through the secretion of specific cytokines and chemokines that provide signals for immune cells both of adaptive and innate immune system. $CD4^+$ T cells recruit and activate $CD8^+$ T cells, macrophages, neutrophils, and other effector cells to sites of infection and inflammation. They help B cells to produce antibodies, to undergo class switching and affinity maturation. They can also act directly on epithelial and mucosal cells during pathogen clearance (for rev.: [157-158].

The terminal differentiation of naïve $CD4^+$ T cells to effector cells is directed by the cytokine milieu in which the T cells get activated when recognizing the antigen, although both the affinity of the peptide-Ag-TCR interaction and co-stimulatory signals influence the differentiation pathway as well.

T helper 1 cells (Th1)

In 1986, Mosmann *et al*. initially proposed a model whereby $CD4^+$ T cells are subdivided into two independent subsets with distinct effector function. Their hypothesis suggested that Th cells can be segregated into a Th1 and Th2 subset on the basis of cytokine expression and bioactivities as well as helper function [159-160].

Th 1 cells mediate immune response against intracellular pathogens. They also activate macrophages, neutrophils or NK cells [161]. They are generated from naïve T cell in the presence of IL-12 and IFNγ. IL-12 is secreted by APCs, mostly by DCs upon TLR activation [162]. IL-12 activates NK cells to produce IFNγ, which in turn activates Stat1 in responding T

cells and leads to upregulation of the master regulator of Th1 cells: T-bet [163]. This transcription factor drives the expression of IL-12 receptor β2 chain and IFNγ [158]. Later also IL-18Rα is upregulated and IL-18 was shown to act synergistically with IL-12 in inducing IFNγ. Except IFNγ, their signature cytokine, Th1 cells produce lymphotoxin α (LTα) and IL-2.

For a long time the Th1 subset was considered to be the pathogenic T cells subset in the context of tissue-specific T cell-driven autoimmune disease and the Th2 subset was thought to be rather protective. This was based on the observations that Th1 cytokines are present in the inflammatory lesions and that they often correlate with disease severity [164]. Another hint came from a study in which EAE was induced by transfer of encephalitogenic Th1 cells [165-166]. However, in stark contrast to this data, the generation and immunisation of mice deficient in Th1 cytokines such as IFNγ and TNFα demonstrated that they were not protected from autoimmune disease. Disease progression and severity of both EAE in TNFα-deficient mice was identical to that in wt mice, while IFNγ- and IFNγR- deficient mice were even hyper-susceptible to the disease [167-168]. In addition, deletion of Th2 cytokines generally did not affect the progression of autoimmune disease [169].

T helper 2 cells (Th2)

Th2 cells mediate host defence against extracellular parasites including helminths. They provide help for B cells to produce IgE antibodies that are effective against parasites and worms. They were also shown to be involved in induction and persistence of asthma [170]. The polarization of Th2 cells is orchestrated by IL-4 and IL-2 [171]. Through signalling of IL-4 Stat6 is activated which leads to upregulation of the master transcription factor GATA-3 [172]. GATA-3 has been reported to induce its own expression and is needed for expression of Th2 cytokines IL-4, IL-5, IL-10 and IL-13 [172-174]. Th2 cells are also able to produce IL-9 and IL-25.

T helper 17 cells (Th17)

Th17 cells were shown to mediate immune response against extracellular bacteria and fungi [175]. Both IL-17A and IL-17F were shown to recruit and activate neutrophils during immune response against those infectious agents. Th17 cells are also connected with the development of autoimmune diseases. One of the paradoxical finding about the role of Th1 cells was the fact that neutralizing or deficiency of IL-12 had the opposite effect than deficiency of IFNγ on the induction of EAE. These studies were based on IL-12 p40-/- mice which are resistant to EAE. The explanation came later when it became clear that p40 can heterodimerise with p19 to form another cytokine, IL-23 [176]. Mice deficient in IL-12p35 were surprisingly not resistant to MOG-induced EAE but actually showed an increased clinical severity [177-179]. It was later shown that the mice deficient in the Il-23 subunit p19 were protected from EAE [180]. Due to the linkage between IL-23 and IL-17 expression a new T helper lineage, Th17, was proposed [181-182]. Studies with IL-17 knockout mice in our laboratory revealed that neither IL-17A nor IL-17F were essential for development of EAE, as these mice were susceptible and only showed minor differences in EAE course compared to control mice. Instead, another cytokine produced by Th cells with upregulated RORγt, namely GM-CSF, was shown to play major role [183-184]; Codarri et al, submitted.

In *in vitro* studies it was shown that TGF-β together with IL-6 induced the development of Th17 cells [185-187]. By signalling of these cytokines, RORγt is induced, which was found to be the master regulator for Th17 subset [188]. Another nuclear receptor RORα is also upregulated in Th17 cells but it is not crucial for IL-17 production [189]. Induction by TGF-β and IL-6 led to the up-regulation of IL-23 receptor and production of IL-21. IL-23 signalling was first considered to be important for Th17 development. Recently it is thought to be critical for the maintenance and restoration of Th17 cell functions [190]. Cytokines secreted by Th17 are IL-17A and F, IL-21 and IL-22. IL-21 was shown to play an important role in

positive feedback regulation for establishing IL-17 producing cells [191-192]. The cytokines that were reported to lead to full Th17 development, IL-6, IL-21 and IL-23, all signal through activation of STAT3 [193].

The role of TGF-β signaling in differentiation and homeostasis of T helper cells

In the majority of mouse with abrogated TGF-β signalling in T cells, these cells undergo hyperproliferation, activation and differentiate into effector cells [49-50, 94, 110]. While effector and memory T cells were highly increased, naïve T cells were found to be strongly reduced. Both CD4 and CD8 T cells up-regulated activation markers such as CD44 and CD69 and adhesion molecules like CD18 and CD11a and as well downregulated CD62L [49]. Furthermore, total numbers of $CD4^+$ and $CD8^+$ T cells were increased in the periphery [49-50]. A BrdU incorporation assay showed increased proliferation of T cells in these models. *In vitro* studies suggested two mechanisms for TGF-β-mediated regulation of T-cell proliferation. TGF-β suppresses IL-2 production of activated T cells, thus inhibiting their proliferation [194].In addition TGF-β controls cell proliferation through regulation of the cell cycle regulators, both inhibitors and promoters [195]. It was shown that Smad3-dependent down-regulation of cdk4 may be essential for in the suppression of T cell proliferation by TGF-β [196]. In the absence of TGF-β produced by T cells these cells also undergo activation and hyperproliferation which suggests that T cells-produced TGF-β is essential to control their homeostasis and maintenance [95].

TGF-β was shown to be not only involved in the regulation of T cell proliferation but also in the control of their differentiation into effector phenotype. An enhanced differentiation of naïve T cells into effector T cells and increased cytokine production was observed in the absence or amelioration of TGF-β signalling in T cells. TGF-β was shown to be able to inhibit Th1 and Th2 cell differentiation *in vitro* through downregulation of the respective transcription factors T-bet and GATA-3 [197-199]. This inhibition was associated with the

TGF-β blockade of TCR-induced activation of Itk kinase, calcium ion influx in T cell and the activation of transcription factor NFAT [200].

In Th2 development TGF-β suppressed IL-4 mediated STAT6 activation [198]. TGF-β had no inhibitory effect on IL-4 and IL-5 production on an established Th2 clone, thus suggesting that TGF-β signalling inhibits differentiation towards that subset, but not acquired effector functions [197]. TGF-β together with IL-6 *in vitro* promotes the differentiation of T cells into Th17 cells [186-187]. The presence of TGF-β appeared to be critical for induction of Th17-defining transcription factor RORγt [201]. In contrast, a recent study claimed that IL-6 and IL-23 together with IL-1β were able to induce full Th17 differentiation in the absence of TGF-β *in vitro*.

Studies performed *in vivo* showed similar changes in T cell differentiation into effector phenotype. In mice expressing dnTGF-βRII under the CD4 promotor, an increased spontaneous differentiation of $CD4^+$ T cells into both Th1 and Th2 cells was reported [197]. However, $CD4^+$ T cells from mice with complete block of TGF-β signalling in T cells differentiated spontaneously exclusively to Th1 cells [50] [49]. The T-bet mediated upregulation of CD122 expression in these cells was suggested by the authors to be responsible for better survival of Th1 cells in the investigated model [49]. In these transgenic animals also the development and maintenance of Th17 cell were investigated. In mice with TGF-βRII-deficient and TGF-β-deficient T cells a strongly reduced Th17 development was observed [187]. The overexpression of TGF-β in T cells enhanced Th17 development *in vivo* [185]. Recently, the induction of the transcription factor RORγt was shown to be Smad2/3 independent, but Smad-dependent TGF-β signalling was still required for a complete Th17 development [202]. Loss of negative regulator of TGF-β signalling Smad7 led to an enhanced Th17 differentiation whereas overexpression of Smad7 reduced this development [203].

SPECIFIC AIMS

The role of TGF-β signalling for $CD4^+$ T cells was studied in many transgenic mouse models with T cells-specific deficiencies for TGF-β1 and its signalling pathways. Constitutive abrogation of TGF-β signalling in T cells has been reported to result in a rapidly lethal autoimmune syndrome in these mice. In addition lack of TGF-β signalling during thymic development resulted in diminished population of $CD8^+$, NK and regulatory T cells [49-51]. Due to impaired T cell development and lethal inflammation in mice with the constitutive abrogation of TGF-β signalling in T cells, it was so far difficult to investigate influence of TGF-β exclusively on $CD4^+$ T cells. To address the role of TGF-β signalling solely for mature $CD4^+$ T cells, we wanted to circumvent the described impact on T cell development and the immediate hyperactivation of the immune system through the use of a novel tamoxifen –inducible CD4-$CreER^{t2}$ strain which we crossed to the $TGFbRII^{fl/fl}$ strain.

The aims of this PhD thesis were:

1. The analysis of the tamoxifen-inducible CD4Cre-ER^{t2} mouse strain in terms of Cre activity and specificity after crossing to two different reporter strains.
2. Investigation of the role of TGF-β signalling in peripheral $CD4^+$ T cells for their homeostasis and maintenance by use of the CD4Cre-ER^{t2} TGF-$\beta RII^{f/f}$ (iCD4TR2) mouse strain.
3. Assessment of the role of TGF-β signalling for the differentiation of T helper cells by use of the iCD4TR2 mouse model.

MATERIAL AND METHODS

Generation of the CD4-CreERt2 allele

Targeting of the Cre-ER^{T2} fusion gene to the CD4 locus was achieved by replacing part of exon 2 including the start codon with the targeting vector *pBluescript CD4-Cre19-ER^{T2}* by homologous recombination in the C57BL/6 derived ES cell line Bruce4. Colonies were analyzed for homologous recombination events by Southern blot analysis of Hind*III* digested genomic ES cell DNA. Two positive clones were used for blastocyst injection. Probes were amplified with: *3' probe:* AAC TGC ACC GTG ACC CTG GAC CAG AAA AAG AA and GTA GGA GTG AAG GTC AGA GAC CAG GAC AAT AG, *5' probe:* CTT CAA ATA ATT AAC AAA ACA ACA AAA CCC TT and AAA AAC CAA AAC CAA CCC AAA CAA AAA ACA T

Animal maintenance

Tgfbr2$^{fl/fl}$ [48] were kindly provided by U. Malipiero and ROSA-EYFP mice [204] were kindly provided by A. Diefenbach, C57BL/6J (B6) mice were purchased from Charles River and congenic C57BL/6-CD45.1 and 2d2 bred in house. CD4CreER^{t2}, CD4Cre, RAGE, TGFβRII$^{fl/fl}$, B6, B6-CD45.1, Rag1$^{-/-}$, ROSA-EYFP mice were maintained in barrier and specific pathogen free facilities at the University of Zurich and handled in accordance with approved protocols under permits of the cantonal veterinary office.

Genotyping

DNA isolation

Biopsies were incubated with tail lysis buffer plus Proteinase K (10 mg/ml) at 55°C for 4 h. After complete digestion the tube was centrifuged for 10 min at 13 000 rpm. To precipitate

genomic DNA the supernatant was decanted into isopropanol and centrifuged again for 10 min at 13000 rpm. The remaining pellet was washed once with 70% ethanol and air dried for 30 minutes. The DNA was solved in TE buffer and stored at 4°C.

Primers for genotyping

All oligonucleotides were purchased from Eurofins MWG Operon (Germany)

Primer	Sequence
CD4CreER^{t2} A	5′-GCCAGCTCATTCCTCCCACTC-3′
CD4CreER^{t2} B	5′-CATGGGACTTTGGGCTTCTAGG-3′
CD4CreER^{t2} D	5′-CCCAACCAACAAGAGAGCTCAAGG-3′
TGF-βRII$^{fl/fl}$ 3	5′-TATGGACTGGCTGCTTTTGTATTC-3′
TGF-βRII$^{fl/fl}$ 4	5′-TGGGGATAGAGGTAGAAAGACATA-3′
2d2 Primer Vα3.2	5′-CCCGGGCAAGGCTCAGCCATGCTCCTG-3′
2d2 Primer Jα18	5′-GCGGCCGCAATTCCCAGAGACATCCCTCC-3′
ROSA 1	5′-AAGACCGCGAAGAGTTTGTC-3′
ROSA 2	5′-AAGGTCGCTCTGAGTTGTTAT-3′
ROSA 3	5′-GGAGCGGGAGAAATGGATATG-3′
EGFP 1	5′-CTATATCATGGCCGACAAGC- 3′
EGFP 2	5′ -ACTGGGTGCTCAGGTAGTGG- 3′
CD4Cre AG-CD4L6	5′ -CCC AAC CAA CAA GAG CTC AAG G- 3′
CD4Cre AG-Cre6	5′ -CCC AGA AAT GCC AGA TTA CG - 3′

Alternatively 2d2 mice were genotyped by use of TCR Vα and Vβ chain specific antibodies: Vα3.2-PE and Vβ11-FITC.

Animal experiments

Tamoxifen application *in vivo*

For tamoxifen (Sigma) application, the substance was first dissolved in 100% ethanol to 1 g/ml, vortexed and mixed with olive oil to a final concentration of 100 mg/ml. The suspension was incubated at 56°C for 15 minutes and sonicated for 20 min. CD4CreER^{t2} x RAGE mice or

$CD4CreER^{t2}$ x $TR2^{fl/fl}$ mice were force-fed with 5mg tamoxifen per day for five consecutive days. Day 7 after start of application is denoted 1 wk p.a.. For long term treatment mice were fed with tamoxifen citrate (Harlan) for 8 weeks at liberty.

Bone marrow chimeras generation

Bone marrow chimeras were generated by i.v. transfer of T cell-depleted bone marrow cells (1×10^7) into lethally irradiated (1100 rad) $Rag1^{-/-}$ mice. After transfer, mice were treated with antibiotics for 14 days; therefore 1 ml Borgal (24%) was added to 250 ml drinking water. Bottles with drinking water were changed every 7 days. Experiment with bone marrow chimeric mice started 5 to 6 weeks post reconstitution.

Thymectomy

Thymectomies were performed under Ketamine/Xylazine anaesthesia according to published procedures [205].

In vivo proliferation

For *in vivo* proliferation analysis BrdU (80mg/100ml) was added to drinking water and changed every second day for 7 days. After cell preparation intracellular staining with anti-BrdU antibody (eBioscience) and Foxp3 Staining Buffer Set (eBioscience) followed by DNAse (Invitrogen) treatment for 1h at 37°C was performed. Samples were analyzed using FACS Canto II.

In vivo suppression assay (induction of colitis)

For the *in vivo* suppression assay Rag1$^{-/-}$ mice were injected intraperitonelly with 4x10^5 conventional T cells (CD45.1$^+$) alone or in combination with 2x10^5 regulatory T cells (CD45.2$^+$). Mice were weighed and assessed for clinical sings of colitis weekly and were killed 9 weeks after transfer. Colons were fixed in 4% formalin, paraffin-cut, and stained with hematoxylin and eosin.

Adoptive transfer

For the adoptive transfer assay Rag1$^{-/-}$ mice were injected intravenously with a 1:1 mixture of 1x10^6 CD45.2$^+$ and CD45.1$^+$ T cells. Expansion of the T cells was assessed by staining of peripheral blood.

Immunizations

Immunization with KLH

For immunizations, mice were injected subcutaneously with KLH (Sigma) together with CFA (100 µg per flank). After 10 days cells from draining lymph nodes and spleen were isolated. They were restimulated with 50µg/ml cognate antigen alone or together with 0.6 mg/ml anti-CD28 or 50µg/ml OVA. Supernatants were collected for ELISA after 48 and 72h. Proliferation of the cells was determined by thymidine incorporation.

Induction of active EAE

For induction of EAE, mice were injected subcutaneously with MOG peptide (GenScript) (100 µg per flank) together with CFA (Difco Laboratories). At day 0 and 2 post immunization mice were injected with 100 µg pertusis toxin (Sigma). Animals were scored for clinical

symptoms of experimental autoimmune encephalomyelitis according to standard operating procedure as following:

Score	Clinics
0	No detectable signs of EAE
0.5	Distal limp tail
1.0	Complete limp tail
1.5	Limp tail and hind limb weakness
2.0	Unilateral partial hind limb paralysis
2.5	Bilateral partial hind limb paralysis
3.0	Complete bilateral hind limb paralysis
3.5	Complete bilateral hind limb paralysis and partial forelimb paralysis
4.0	Moribund (mouse completely paralyzed)
5.0	Dead

Transfer of T cell and induction of EAE

Mice were treated with tamoxifen as described above. 2 weeks p.a. lymphocytes were isolated and injected i.v. into $RAG1^{-/-}$ animals. Recipient mice were immunized with MOG peptide as described above.

Passive EAE (Adoptive transfer of EAE)

For adoptive transfer mice were treated with tamoxifen as described above. The animals were immunized with MOG (as described above); 100 µg Pertussis Toxin were injected i.p. on the day of immunization (day 0). Mice were euthanized 7 days after immunization and lymphocytes from spleen and draining lymph nodes were isolated. Cells were expanded by plating 7-8 million/ml in culture dishes (BD Falcon) together with 10 µg/ml MOG and 10 ng/ml IL-23, and cultured for two days. $RAG1^{-/-}$ animals were injected i.p. with 20 million cells.

Histological Tissue Analyses

Mice were euthanized with CO2, perfused with PBS and 4% paraformaldehyde in PBS. For histological analysis kidney, liver, heart, colon, small intestine were fixed in 4% paraformaldehyde in PBS, paraffin-embeded, cut into 30 µm thick sections and stained with hematoxylin-eosin according to standard procedures. For TUNEL staining 4 µm thick paraffin sections underwent TdT (terminyl deoxynucleodityl transferase, Roche Diagnostics)-mediated biotin-dUTP end nick labeling reaction for 30 min at 37°C. Digoxigenin was visualised by anti-Digoxigenin (Sigma) and Intense R HRP (Leica). The sections were counterstained with hematoxyline.

Quantitative real time PCR analysis

Different subsets of $CD4^+$ T cells were isolated by FACS sorting and used for mRNA extraction (RNeasy mini kit, Qiagen). mRNA was transcribed with M-MLV reverse transcriptase (Invitrogen). Quantitive RT-PCR was performed with MyIQ cycler (Biorad) using SyberGreen (Invitrogen), following primers were used:

Primers for qRT-PCR	**Sequence**
TGF-β1	5′-GACGTCACTGGAGTTGTACC-3′
	5′-GGTTCATGTCATGGATGGTGC-3′
TGF-βRI	5′-CATTCACCACCGTGTGCCAAATGA-3′
	5′-ACCTGATCCAGACCCTGATGTTGTT-3′
TGF-βRII	5′-AACGACTTGACCTGTTGCCTGT-3′
	5′-CTTCCGGGGCCATGTATCTT-3′
RNA polymerase 2	5′-CTGGTCCTTCGAATCCGCATC-3′
	5′-GCTCGATACCCTGCAGGGTCA-3′

Cell culture

All cells were handled under sterile conditions in a laminar flow bench. Cells were cultured at 37°C in a 5 % CO_2 atmosphere. Lymphocytes were cultured in RPMI 1640 medium, supplemented with 10 % FCS, 1 % Penicillin-Streptomycin and 0.5 % β-Mercaptoethanol.

In vitro proliferation and apoptosis assays

Lymphocytes from LN and spleens were stained for CD4, CD44, CD62L, CD25, CD45RB and sorted by using FACS Aria. The purity for each population was above 95 %. Cells were cultured in AIM-V medium (Invitrogen) supplemented with 0.05% 2-mercaptoethanol (Sigma) without stimulation. After 20 and 40h staining for Annexin V (BD Bioscience) and Topro3 or propidium iodide (1µg/ml) was performed. For CFSE-labelling cells from the spleen and lymph nodes were stained for 20 minutes in the dark with CFSE (carboxyfluorescein diacetate succinimidyl diester, 5 µM) washed in PBS and cultured in RPMI 1640 (Invitrogen) medium supplemented with 10% FCS, 1% penicillin-streptomycin, 0.5% 2-mercaptoethanol. Cells were stimulated for 72h with 5 µg/ml CD3 (2C11) and 5 µg/ml CD28 (N37).

In vitro suppression assay

Sorted conventional T cells (5×10^4) and T_{reg} cells (in ratios according to figure legend) were cultured for 96 h in round-bottom plates along with anti-CD3 antibody (2C11, 2 µg/ml) and irradiated splenocytes in RPMI 1640 medium supplemented with 10% FCS, 1% penicillin-streptomycin, 0.5% 2-mercaptoethanol. T cell proliferation was determined by thymidine incorporation and CFSE-labelling of T_{conv} cells as described above.

In vitro T cell skewing conditions

For skewing experiments lymph node cells and splenocytes were pooled and plated in complete RPMI in 24-well flat-bottom plates (BD Falcon) at a density of 4 million cells/ml per well. Cytokines and antibodies were added according to following protocols. Cells were cultured for 3-5 days.

Th1 condition

αCD3	5 µg/ml
αCD28	5 µg/ml
IFNγ	20 ng/ml
IL-12	20 ng/ml
IL-2	10 ng/ml

In order to perform intracellular staining on day 3 of culture, the cells were restimulated with 50 ng/ml PMA, 500 ng/ml Ionomycin and 1 µl/ml BD GolgiPlug for 5 h.

Th2 condition

αCD3	5 µg/ml
αIFNγ	10 µg/ml
αIL-12	10 µg/ml
IL-4	25 ng/ml
IL-2	10 ng/ml

On day 3 cells were split 1:2, fresh medium was added up to a total volume of 1 ml per well. IL-2 and IL-4 were added again. Cells were restimulated as described above and harvested for intracellular staining either day 4 or day 5, supernatants were frozen at -20°C to perform ELISA for IL-5 and IL-10 production.

Th17 condition

αCD3	5 µg/ml
αCD28	5 µg/ml
αIFNγ	10 µg/ml
TGF-β	10 ng/ml
IL-6	20 ng/ml

On day 3 cells were split 1:2, fresh medium was added up to a total volume of 1 ml per well. αIFNγ, TGF-β and IL-6 was added again in the mentioned concentrations. Intracellular staining was performed either day 4 or 5 after restimulation.

For control conditions, cells were either cultured in pure complete RPMI 1640 or αCD3 and αCD28 was added accordingly:

Control condition

αCD3	5 µg/ml
αCD28	5 µg/ml

Cells were harvested after restimulation with PMA, Ionomycin and GolgiPlug as described above.

In order to delete the TGF-βRII *in vitro* 4-OH Tamoxifen was added in a final concentration of 1 µM.

Flow Cytometric Analyses

For flow cytometry the following fluorochrome conjugated antibodies were used: anti-CD3, anti-CD4 (L3T4), anti-CD8 (53-6.7), anti-CD11b, anti-CD11c, anti-CD19, anti-CD25, anti-CD44, anti-45, anti-CD45.1, anti-CD45.2, anti-CD45RB, anti-CD49b, anti-CD62L, anti-CD69, anti-CD122, anti-CD154, anti-GITR, anti-NK1.1, anti-IL2 all purchased from BD Biosciences, anti-TGF-βRII from R&D, anti-Ly6A/E, anti-CD90.2 from BioLegend anti-Foxp3, anti-BrdU, anti-NKp46 from eBiosciences. Staining for transcription factors Foxp3, Tbet and GATA3 was performed using Foxp3 Staining Buffer Set (eBiosciences) according to manufacturer's protocol.

For cytokine staining following fluorochrome conjugated antibodies were used: anti-IFNγ, anti-17A, anti-IL5, anti-GM-CSF from BD Biosciences; anti-IL4 from eBiosciences. For intracellular cytokine staining cells were stimulated for 4 to 6 h with 50 ng/ml PMA and 500 ng/ml Ionomycin and the 1 µl/ml GolgiPlug (BD Bioscience)

Samples were acquired using FACS CantoII (BD Biosciences) and analyzed with FlowJo software (Treestar).

To enrich the $CD4^+$ T cells population magnetic sorting was performed (CD4 T cell isolation kit, Milteney Biotech) according to manufacturer's protocol.

T_{reg} cells and T_{conv} cells were sorted on a FACS Aria (BD Biosciences) on the basis of being $CD4^+CD45RB^{lo}CD25^+$ and $CD4^+CD45RB^{hi}CD25^-$ respectively. Naïve CD4 T cells were sorted on the basis of being $CD4^+CD44^{hi}CD62Ll^{lo}$, effector memory $CD4^+CD44^{lo}CD62L^{hi}$, central memory $CD4^+CD44^{hi}CD62L^{hi}$ and effector $CD4^+CD44^{lo}CD62L^{lo}$.

Enzyme-linked immunosorbant assay (ELISA)

For the detection of serum antibody levels ELISA plates (PVC, BD Falcon) were coated with antibodies (anti-IgA, anti-IgG2a, anti-IgM) from Southern Biotech, for detection secondary antibodies (anti-mouse IgM, IgG, IgA peroxidase conjugated) from Sigma were used. The concentrations of cytokines in the culture supernatants were determined by ELISA (INFγ, IL-4, GM-CSF, IL-10, and IL-2, IL-17 from BD Biosciences) according to manufacturer's protocols.

For detection of anti-dsDNA antibodies from the serum, ELISA plates (BD Falcon) were pre-treated with 0.1% poly-L-lysine (Sigma) for 2 h, coated with 100 μg/ml DNA (Sigma) over night, blocked with 2% BSA for 2h. After washing with PBS anti-mouse IgG γ chain HPR conjugated antibody (Sigma) was applied for 45 min and developed with stabilised chromogen (Invitrogen).

ELISPOT

For ELISPOT , cells from lymph nodes of KLH immunized mice ($2x10^5$ cells/well) were plated in complete RPMI medium containing 50 μg/ml of KLH in 96-well plates (Millipore) coated with 7.5 μg/ml of anti-IFN-γ (AN18; Mabtech) Plates were incubated at 37 °C in 5% CO_2 for 20h. Then, 0.5 μg/ml of biotin-conjugated anti-IFN-γ (R4-6A2; Mabtech) was added, followed by incubation at 25 °C for 2 h or 4 h, respectively. After plates were washed, streptavidin–alkaline phosphatase (Mabtech) was added, followed by incubation for 1 h at 25 °C. Substrate solution BCIP/NBTplus (5-bromo-4-chloro-3-indolylphosphate–nitro blue tetrazolium; Biosource) was added to the wells, which were developed until distinct spots emerged. Plates were analyzed with an enzyme-linked immunospot reader (ImmunoSpot; CTL).

Immunofluorescent staining

NIH3T3 cells were cultured on poly-d-lysine (Sigma) precoated glass slides. Cells were fixed with methanol (Fluka) for 10 minutes and permeabilized with 1% Triton X-100 (Sigma) for 20 minutes. After blocking with 10% goat serum (Dako), cells were incubated over night with serum diluted 1:200 in 1% goat serum and 0.2% Triton X-100 (Sigma). Cells were incubated with goat anti-mouse IgG antibody AlexaFluor 546 conjugated (Invitrogen) for 45 min and the nuclei were counterstained with Hoechst 33342 (Invitrogen). Analysis was performed on 40 mm microscope with CellM software.

Statistical analysis

P values were calculated with Student's t-test using Prism software. P values of less than 0.05 were considered significant.

RESULTS

The role of TGF-β signalling in T cells was studied in many transgenic mouse models. The studies performed with the strain in which TR2 was deleted from T cells already at the DP stage of thymic development [49-50] showed the role of TGF-β in development, maintenance and differentiation of many $CD4^+$ T cell subsets. However, in this model development of both $CD4^+$ and $CD8^+$ T cells is affected and the mice suffer from severe autoimmune disease which leads to their death at the age of few weeks. It is difficult to say if some changes observed in $CD4^+$ T cell compartment in these mice are caused by sickness or if it is a primary effect of the absence of TGF-β signalling. To more specifically address the role of TGF-β signalling solely for mature $CD4^+$ T cells, we therefore circumvented the described impact on T cell development and the immediate hyperactivation of the immune system through the generation of a CD4-$CreER^{t2}$.

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Generation and characterization of the CD4-$CreER^{t2}$ strain

The inducible CD4-$CreER^{t2}$ knock-in strain was generated by Saskia Hemmers at the Institute for Genetics, University of Cologne. Tamoxifen-inducible Cre activity in this strain was expected to target mostly to $CD4^+$ T cells (**Fig. 1 a,b**) [206]. Inducibility and specificity of Cre activity in the CD4CreERt2 stain was first assessed by crossing to the RAGE reporter strain featuring Cre-facilitated EGFP expression [207] and ROSA-EYFP strain [204]. Different doses and application routes of tamoxifen were tested including i.p injection and gavage. The highest EGFP expression was achieved after oral application of tamoxifen for five consecutive days. The reporter protein was detected in up to 70% of the peripheral $CD4^+$ T cells. No EGFP signal was measured in $CD8^+$ T cells, B lymphocytes or other CD4 negative immune cells. Also in the absence of tamoxifen application EGFP was not detectable

in $CD4^+$ T cells. In the double positive stage of T cell development hardly any recombination could be detected (**Fig. 1c** and data not shown).

As anticipated, we also observed recombination in a small fraction of $CD4^+$ splenic dendritic cells (DCs) and $CD4^+$ positive lymphoid tissue inducer (Lti) cells (**Fig. 1 d**). To obtain a transgenic locus even closer to the physiological situation we next removed the neomycin resistance gene (Neo(R)) by *in vivo* FLP-mediated recombination ([208]. Unexpectedly, yet as described for another Cre transgenic mouse strain [209], removal of Neo(R) decreased Cre recombination frequency (F**ig. 1 e**) without improving specificity. In all further experiments we therefore used animals bearing the Neo(R)-containing allele.

To achieve recombination *in vitro* 4-Hydroxytamoxifen, which is a metabolite of tamoxifen, was used. Different concentrations of this ligand were tested on unstimulated or $CD4^+$ T cells activated with PMA/Ionomycin and Concanavalin A. The percentage of recombination upon 4-OH tamoxifen application was lower than after *in vivo* treatment with tamoxifen reaching 60% of activated CD4 T cells (**Fig. 2a,b**). Similar to *in vivo* treatment no recombination in $CD8^+$ or non-T cell compartment was found (data not shown). No increase in cell death of stimulated T cells was observed after 4-OH tamoxifen treatment.

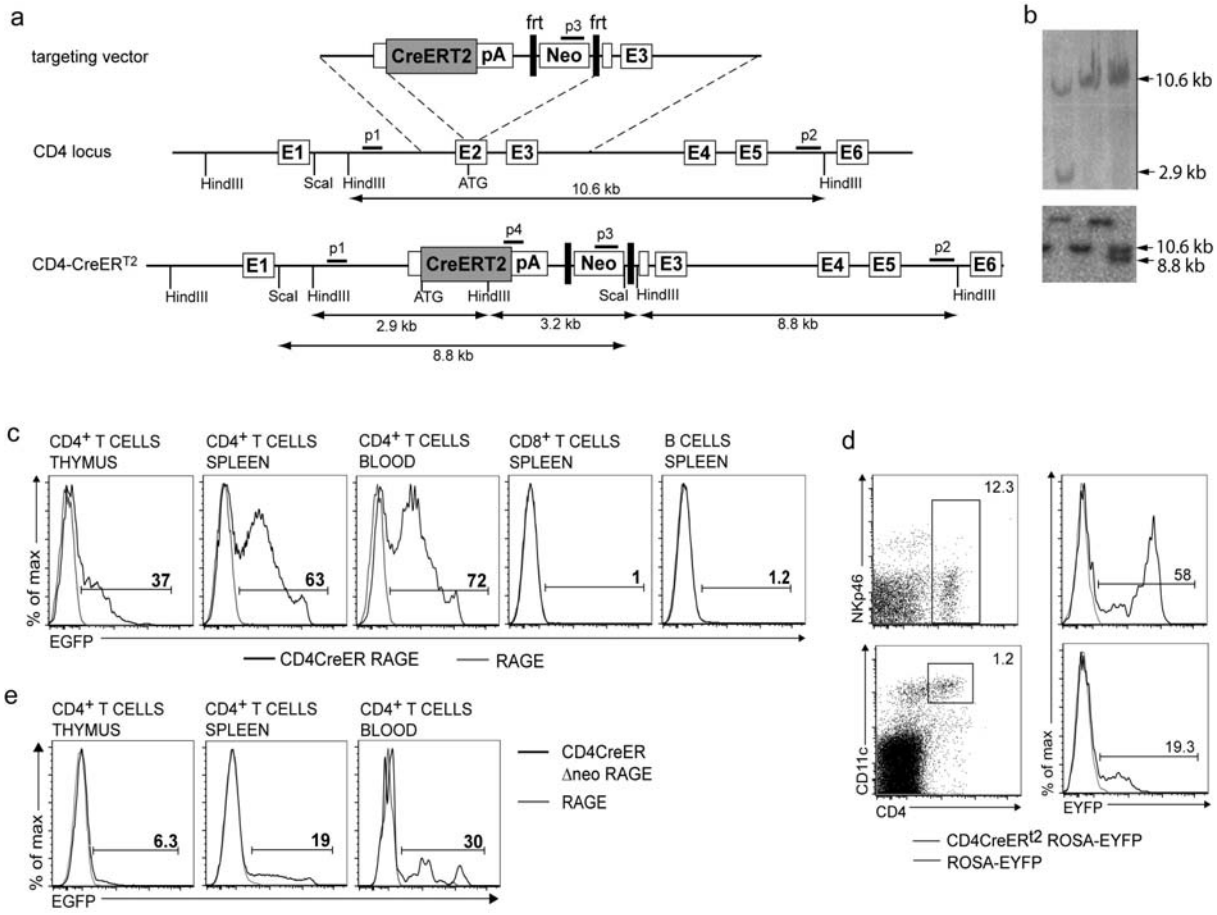

Fig. 1 Targeting strategy and primary analysis of the CD4CreER^{t2} system

(a) Schematic map of the targeting strategy for the CD4 Locus. The Cre-ER^{T2} open reading frame and an FRT-flanked neomycin resistance gene were inserted into exon 2 of the CD4 locus of murine ES cells. HindIII restriction sites used for Southern blot analysis of the targeted ES cell are indicated. (b) Southern Blot screen of the targeted ES cells after digestion with HindIII and hybridization with the 5' external probe and 3' external probes. Homologous recombination is indicated by newly appearing 2.9 kb and 8.8 kb bands for the targeted allele in addition to the 10.6 kb band for the WT allele. (c) Flow cytometric analysis of EGFP expression after *in vivo* administration of tamoxifen. CD4-CreER^{t2}/RAGE mice were treated with tamoxifen orally for 5 consecutive days and analysed at day 7 p.a.. These data are representative results of four independent experiments. (d) Flow cytometric analysis of EYFP expression after *in vivo* administration of tamoxifen. CD4-CreER^{t2}/ROSA-EYFP mice were treated with tamoxifen orally for 5 consecutive days and analysed at day 7 p.a.. The gating scheme is indicated. These data are representative results of two independent experiments. (e) Flow cytometric analysis of EGFP expression after *in vivo* administration of tamoxifen. CD4-CreER$^{t2\Delta Neo}$/RAGE mice were treated with tamoxifen orally for 5 consecutive days and analysed at day 7 p.a.. These data are representative results of two independent experiments

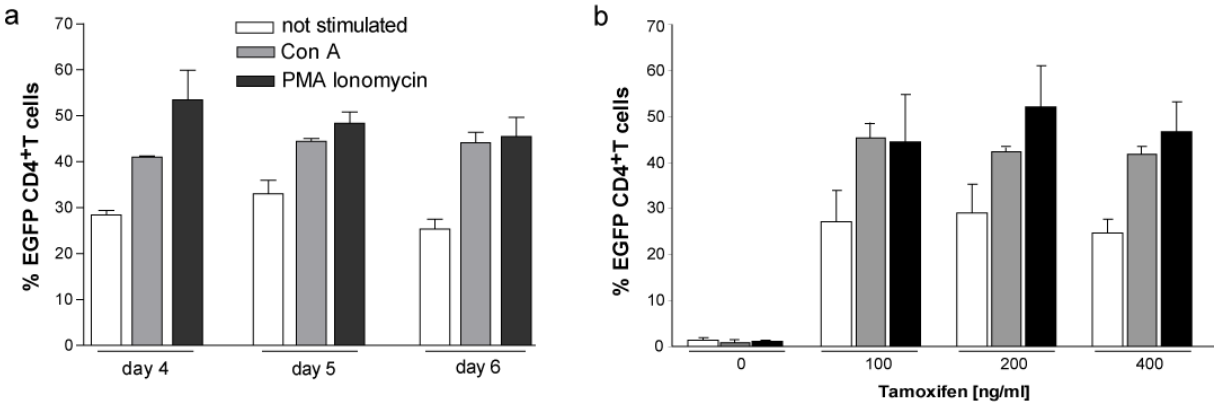

Fig. 2 *In vitro* analysis of the CD4CreER^{t2} system in CD4CreER^{t2}EGFP mice

(a) The percentage of EGFP^{+} CD4 T cells at different time points after *in vitro* culture with 4-hydroxytamoxifen. (b) The percentage of EGFP^{+} CD4^{+} T cells at day 4 of culture with different concentrations of 4-hydroxytamoxifen

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Efficient ablation of TR2 from peripheral T cells without affecting T cell development

Previous genetic analyses of the function of TGF-β1 for T cells have either relied on germline gene deficiencies or involved conditional mutagenesis by use of the lck-cre, CD4-cre, and MX-cre transgenes, thus resulting in gene ablation at the CD4^{-}CD8^{-} (double negative) and CD4^{+}CD8^{+} (double positive) stages of thymic development and during hematopoiesis, respectively. Consequently, these models were characterized by alterations in thymic development of all T cell lineages [49-51] possibly causing the development of the observed autoimmune syndromes.

Since we sought to investigate the role of TGF-β signalling for peripheral T helper cells we then crossed the CD4-CreER^{t2} and a conditional TR2 (TR2^{f}) allele [210] obtaining CD4-CreER^{t2}/TR2$^{f/f}$ (iCD4TR2). Throughout all experiments we used tamoxifen-treated CD4-CreER^{t2}/TR2$^{f/+}$and TR2$^{f/f}$ as well as untreated iCD4TR2 mice as genetic and treatment controls. In none of the experiments we found differences between these control groups and unless indicated otherwise they are presented as one control. First, we tested ablation of TR2

from $CD4^+$ T cells by tamoxifen treatment of iCD4TR2 mice for 5 days (herein called tamiCD4TR2) and, consistent with our observations using the reporter strain found surface TR2 to be reduced from 80% to 10% on blood $CD4^+$ T cells 1 wk p.a. (**Fig. 3a**). Expression of TR2 by other cell types including $CD8^+$ T cells within tamiCD4TR2 mice was unaffected and similar to that of control mice (**Fig. 3a** and data not shown**)**. Target allele recombination was therefore efficient and restricted to $CD4^+$ T cells. Next, we used quantitative RT-PCR to analyze the TR2 mRNA levels within T cell subsets 14 days after tamoxifen treatment. Sorted naïve (T_n, $CD62L^{hi}$ and $CD44^{lo}$), central memory ($CD62L^{hi}$ and $CD44^{hi}$), effector ($CD62L^{lo}$ and $CD44^{lo}$), effector memory (T_{efm}, $CD62L^{lo}$ and $CD44^{hi}$) and regulatory T cells (T_{reg}, $CD25^+CD45RB^{hi}$) from tamiCD4TR2 mice had on average a 19-fold reduction of TR2 mRNA levels, thus confirming successful and comparable deletion of the receptor (**Fig. 3b**) in the investigated T cell subpopulations. To confirm the physiological relevance of the observed TR2 deletion we performed an *in vitro* Th17 differentiation assay with cells from tamiTR2 and control animals. Absence of TR2 expression resulted in almost complete loss of Th17 and RORγt expression, thus confirming efficient abrogation of TGF-β signalling in $CD4^+$ T cells (**Fig. 3c**).

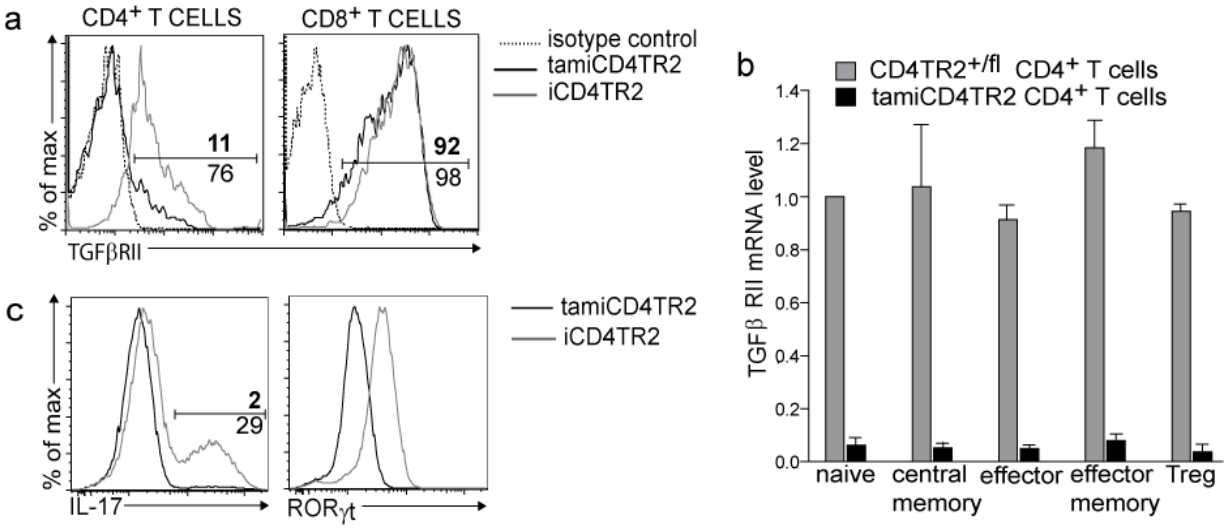


Fig. 3 Efficient deletion of TR2 from peripheral CD4$^+$ T cells.

(a) Flow cytometric analysis of TR2 expression by CD4$^+$ and CD8$^+$ T cells from peripheral blood. These data are representative results of four independent experiments (b) Expression level of TR2 mRNA in different FACS-sorted splenic CD4$^+$ T cell subsets determined by quantitive RT-PCR. These data are representative results of three independent experiments. (c) Flow cytometric analysis of the expression of IL17 and RORγt by CD4$^+$ T cells after 4 days of culture in Th17 skewing conditions.

Because CD4 expression starts at the thymic DP stage we investigated the extent and consequences of TR2 ablation in the thymus. Analysis of thymic cellularity, size of the CD4$^+$CD8$^+$ (DP), CD4$^-$CD8$^-$ (DN), CD4$^+$ and CD8$^+$ (SP) populations or expression of the markers CD5, CD24, CD62L, CD69 revealed no differences between experimental and control groups (**Fig.4** and data not shown). The number and phenotype of thymic T_{reg} cells was found to be unmodified as well (**Fig. 4** and data not shown). Thus, in contrast to previous models used for analysis of TGF-β function in T cells, our novel mouse strain allows the study of TGF-β function in mature T cells without a disturbed thymic T cell development.

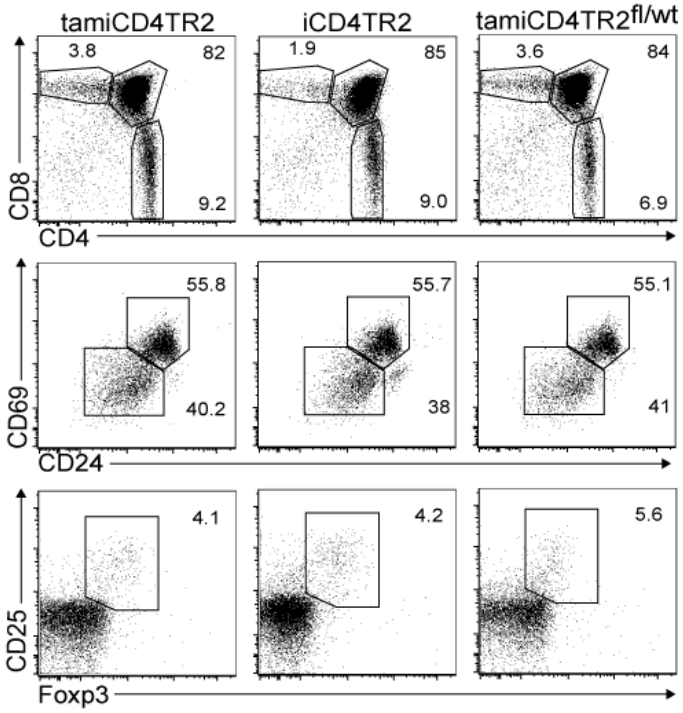


Fig. 4 Thymic development is not affected in tamiCD4TR2 mice

Expression of CD4 and CD8 on thymocytes (upper panel), CD69 and CD24 markers on $CD4^+$ SP thymocytes (middle panel), Foxp3 and CD25 on $CD4^+$ SP thymocytes (lower panel) isolated from tamiCD4TR2 and control mice at 2 weeks p.a.. These data are representative results of three independent experiments.

Peripheral abrogation of TR2 signalling in $CD4^+$ T cells does not lead to autoimmunity

Constitutive ablation of TGF-β signalling during thymic development invariably results in a generalised and rapidly lethal autoimmune disorder [49-50]. We therefore investigated the effect of removal of TR2 from peripheral T cells by following clinical appearance, weight, autoantibodies, and histopathology in comparison to control animals. Unexpectedly and in contrast to all previously reported systems of ablation of TGF-β signalling in T cells tamiCD4TR2 mice appeared healthy without any signs of autoimmunity two and four weeks p.a.. To exclude that the lack of autoimmune inflammation was due to the short tamoxifen treatment we performed an extended experiment in which we fed animals with tamoxifen

citrate for two months and followed them for three additional months. Similar to the short-term treatment, we did not observe any signs of disease, neither clinically nor by weight loss (**Fig. 5a**). Because mild inflammation may not present clinically we performed histopathological analysis of liver, kidney, pancreas, heart and thyroid gland but could not detect any autoimmune infiltrates in any of these organs (data not shown). We then assessed whether the absence of TR2 on peripheral $CD4^+$ T cells would lead to a secondary deregulation of B cell tolerance and to the consequent production of autoantibodies as observed in another model [49]. Yet neither by immunofluorescence staining of NIH 3T3 cells with sera from tamiCD4TR2 mice treated with tamoxifen for five days or tamoxifen citrate for eight weeks nor by ELISA against dsDNA could we detect production of significant levels of autoantibodies (**Fig. 5b, e**). Thus, removal of TR2 from mature $CD4^+$ T cells seems not to result in tolerance loss and autoimmunity.

One possible explanation for this unexpected outcome is that thymic T cell output could compete with and dilute out $CD4^+$ T cells lacking TR2 in our model. To exclude this we thymectomized iCD4TR2 and control mice one week before an 8-week tamoxifen citrate treatment. Even in the absence of newly developing thymic T cells, we again failed to observe any signs of autoimmune disease by clinical appearance and weight up to five months p.a. (**Fig. 5c**). Total serum antibody titers were indistinguishable between thymectomized tamiCD4TR2 and control animals and autoantibodies were undetectable in sera from both groups (**Fig. 5d**). Again, histopathology of various organs did not reveal any indications of immune infiltrates (**Fig. 5f** and data not shown). In conclusion and in contrast to previous reports [49-50, 110, 120], ablation of TR2 from peripheral T cells does not result in impaired T and B cell tolerance or autoimmunity.

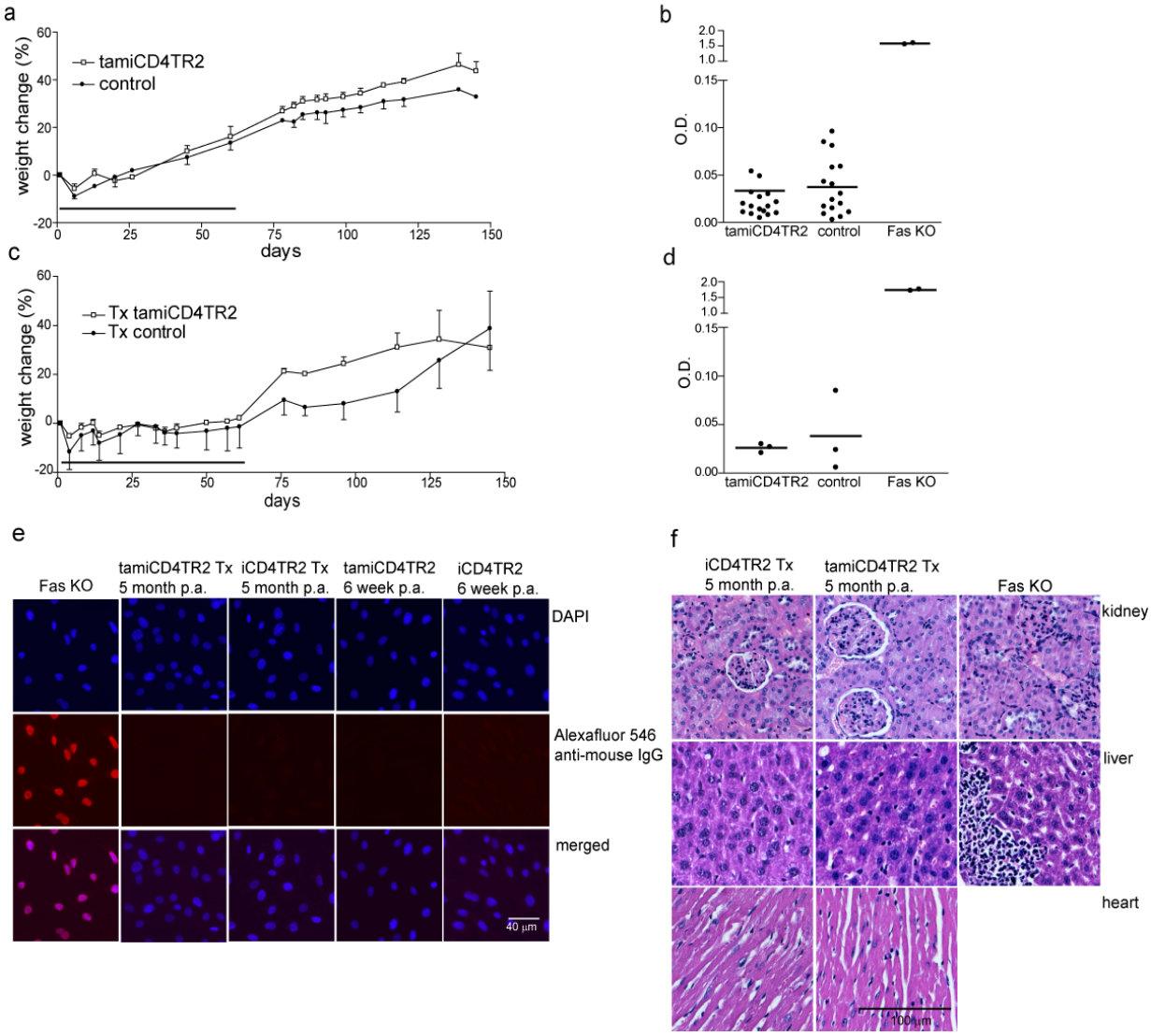


Fig. 5 Lack of autoimmunity after abrogation of TR2 signalling in CD4 T cells in adult mice

(a) iCD4TR2 and control mice were treated with tamoxifen citrate for two months and kept for another three months on normal diet. Body weight was monitored during whole period of experiment (mean ± SEM, 5 mice per group, representative data of two independent experiments). (b) ELISA for anti-dsDNA antibodies in sera from tamiCD4TR2 and control mice after 2, 4, 6 weeks and 5 month p.a.; as positive control a serum from a *fas*-deficient mouse was used. (c) Repetition of the long term tamoxifen citrate treatment experiment as in a), but mice were thymectomized 1 week before tamoxifen treatment started (mean ± SEM, 3 mice per group, representative data of two independent experiments). (d) Anti-dsDNA ELISA as in b) but with sera from thymectomized mice after 4 month p.a. (e) Detection of antinuclear antibodies by immunofluorescent staining of NIH3T3 cells with sera from indicated mice. Representative micrographs are shown. The size bar indicates 40 µm (f) Representative micrographs of H&E-stained tissue sections of indicated organs isolated from thymectomized tamiCD4TR2 or control mice after long tamoxifen treatment (40x) The size bar indicates 100 µm.

Non-conventional NK T cells are not generated upon peripheral removal of TR2

Although absence of TGF-β signaling in T cells was shown to block NK T cell development in the thymus [50-51], it also leads to the appearance of an aberrant non-conventional NK T cell population in the periphery. These $CD8^{low}$ or $CD4^{+}$ cells contain most of the autoaggressive repertoire and do not recognize CD1/αGalCer [50]. We therefore analysed whether such unconventional NK T cells were also found upon removal of TR2 from peripheral $CD4^{+}$ T cells. In contrast to CD4-cre/$TR2^{fl/fl}$ mice we did not find any expansions of $CD8^{low}$ or $CD4^{+}$ $NK1.1^{+}$ T cells in tamiTR2 compared to control mice two and four weeks p.a. (**Fig. 6**). Even five months p.a. and after thymectomy these cells had not accumulated (**Fig. 6**). Thus, it seems that the pathogenic $CD8^{low}$ or $CD4^{+}$ NK T cell populations develop only when TGF-β signalling is compromised during thymic development.

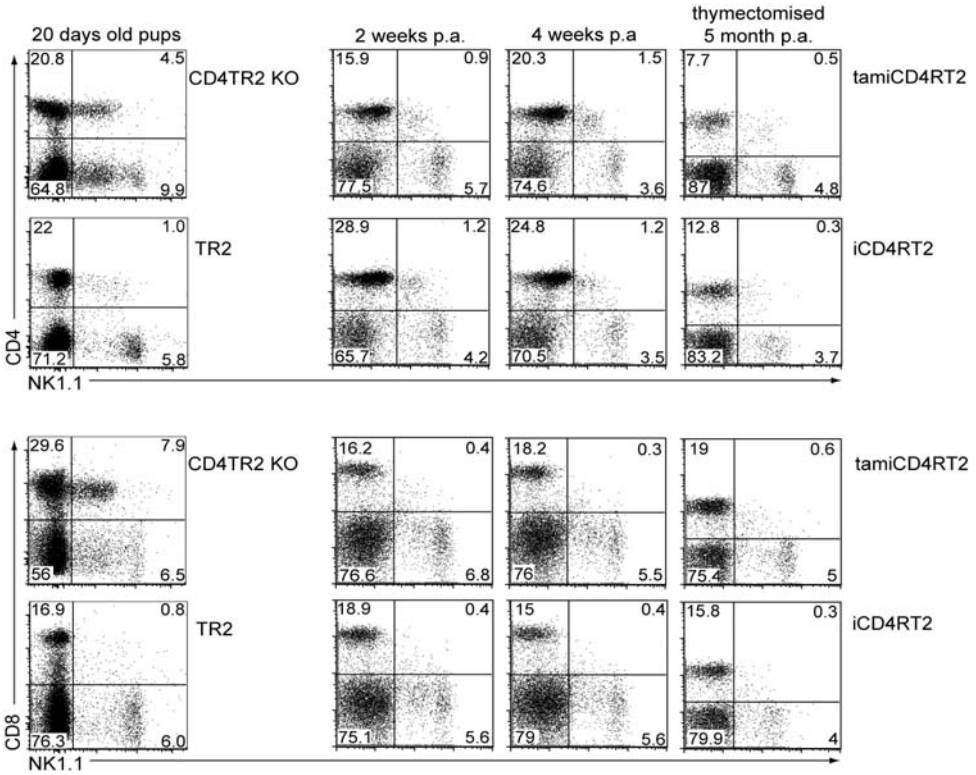


Fig. 6 Normal NK1.1 positive T cell subpopulations in tamiCD4TR2 mice

Flow cytometric analysis of NK1.1 expression on splenic $CD4^{+}$ and $CD8^{+}$ T cells isolated from tamiCD4CreTR2 or control mice after different time points post tamoxifen administration. As a positive control 20 days old CD4CreTR2 pups were used.

Dysregulated homeostasis of CD4$^+$ T cell subpopulations in the absence of TGF-β signalling

All reported models of modified TGF-β signalling in T cells have shown dysregulation of T cell activation, massively increased proliferation and concomitant decrease in the number of T_n cells [49-50]. When we compared tamiCD4TR2 to control mice at 2 and 4 wks p.a. we found CD4$^+$ but not CD8$^+$ T cell numbers in spleens and LNs slightly reduced (**Fig. 7a**). The total number and fraction of T_n cells in spleen were decreased 2 and 4 weeks p.a. (**Fig. 7b,c**) while an opposite effect in percentage and total numbers was observed for T_{efm} and T_{reg} cell compartments with significant expansions 2 and 4 weeks p.a. (**Fig. 7b,c**). At all time points the percentages and total numbers of central memory and effector CD4$^+$ T cells remained unaffected in tamiCD4TR2 mice (data not shown). Interestingly, six weeks p.a. the observed differences had altogether disappeared (**Fig. 7b,c**), indicating that the effect of TR2 removal on population sizes was transient.

Differences in cell numbers result either from changed proliferation or apoptosis rates. To distinguish these possibilities we first assessed proliferation by BrdU incorporation. We found that the fraction of proliferating T_{efm} and T_{reg} cells had increased sharply 2 weeks p.a. in tamiCD4TR2 compared to control animals while the fraction of cycling T_n and central memory CD4$^+$ T cells remained unchanged (**Fig. 7d** and data not shown).

To exclude thymic T cell replenishment we again performed thymectomy before removing TR2. The phenotype observed in not thymectomised tamiCD4TR2 mice now persisted at time point 6 weeks p.a, with significantly smaller CD4$^+$ T_n and larger T_{efm} cells populations (**Fig. 8 a,b**). Thymectomised tamiCD4TR2 and control mice were also analysed after longer period, at 20 weeks p.a. and the percentage and number of CD4$^+$ T_n decreased even further.

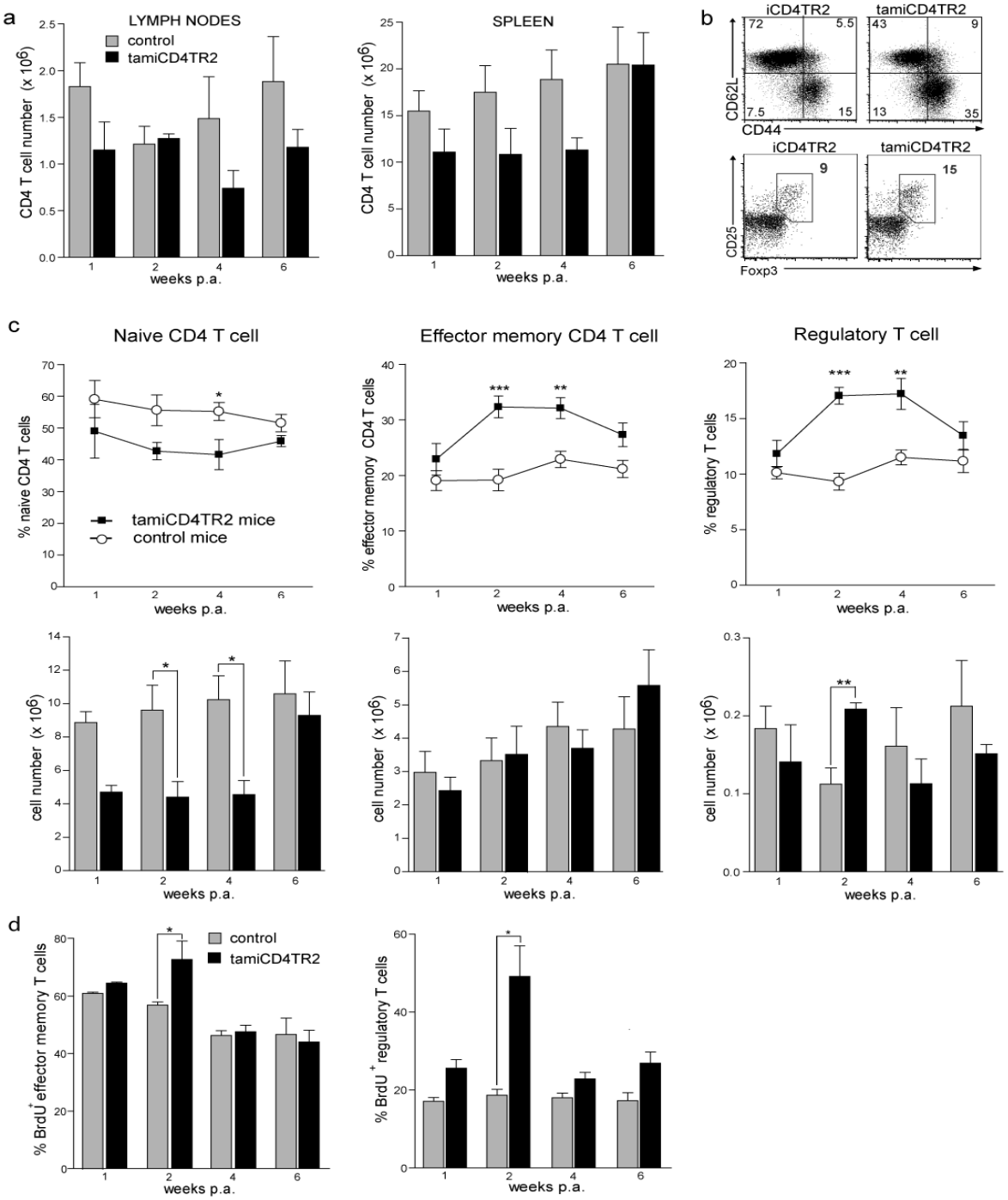


Fig. 7 Increased proliferation of T_{efm} and T_{reg} cells upon removal of TR2

(a) Absolute number of $CD4^+$ T cells in spleens and LN of tamiCD4TR2 and control mice 1, 2, 4 and 6 weeks p.a. Mice were treated with tamoxifen for 5 consecutive days (mean ± SEM, 9 mice per group, analysed in three independent experiments). (b) Flow cytometric expression analysis of CD62L, CD44, CD25, Foxp3 on $CD4^+$ splenic T cells of tamiCD4TR2 and control mice at 2 weeks p.a.. These data are representative results of three independent experiments. (c) The percentage of T_n, T_{efm}, and T_{reg} cells in the spleen of tamiCD4TR2 and control mice (percentage out of $CD4^+$ T cell, mean ± SEM, 9 mice per group, analysed in three independent experiments). (d) The percentages of $BrdU^+$ T_{efm} and T_{reg} CD4 T cells isolated from spleen of tamiCD4TR2 and control mice, gated on $CD4^+$ T cells (mean ± SEM, 9 mice per group, analysed in three independent experiments). (e) The percentage of T_n, T_{efm} and T_{reg} cells in the spleen of thymectomised tamiCD4TR2 and control mice (mean ± SEM, 9 mice per group, analysed in two independent experiments).

Although the T_{reg} cell population in thymectomized tamiCD4TR2 mice appeared to be slightly enlarged at this time point, this was not statistically significant. The absolute numbers of T_n, T_{efm}, or T_{reg} showed similar tendencies **(Fig. 8 a,b)**.

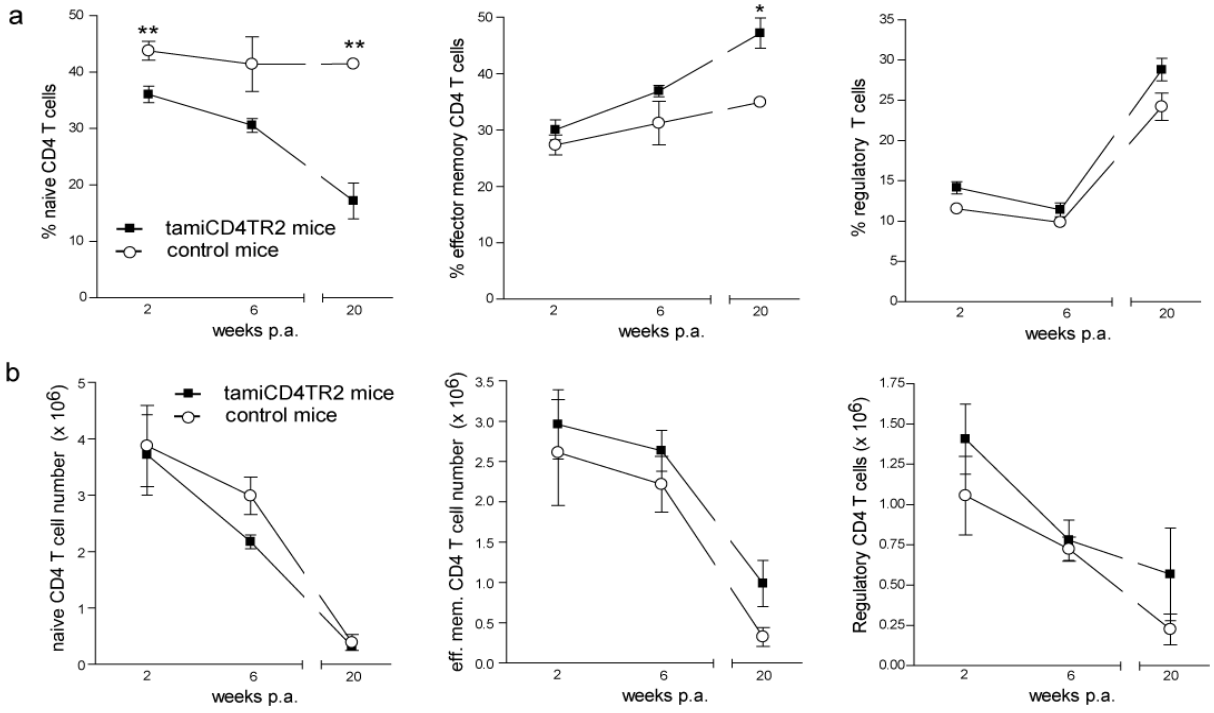


Fig. 8 Composition of CD4 T cell compartment in thymectomise mice

(a) The percentage of T_n, T_{efm}, and T_{reg} cells in the spleen of thymectomised tamiCD4TR2 and control mice (percentage out of $CD4^+$ T cell, mean ± SEM, 9 mice per group, analysed in three independent experiments) (b) Absolute number of T_n, T_{efm} and T_{reg} cells isolated from spleen of thymectomised tamiCD4RT2 and control (mean ± SEM, 9 mice per group, analysed in three independent experiments).

We tested whether known factors governing T cell survival and proliferation were changed in the absence of TR2 and contributed to the increase proliferation of T_{efm} and T_{reg} cells. No differences in expression of CD25 or IL-2 production between tamiCD4TR2 and control mice were found while a slight increase of CD122 (IL-2Rβ) on $CD4^+$ tamiCD4TR2 T cells was detected 2 week p.a. (**Fig. 9a, b**).

Next, to test for enhanced susceptibility to apoptosis by cells lacking TR2, it was removed from splenic $CD4^+$ T cells *in vitro*. Annexin V and Topro-3 staining after 20 and 45h showed strongly increased incidence of apoptosis of cells lacking TR2 in comparison to control cells (**Fig. 9c**). Also, TUNEL staining performed on splenic sections revealed the profound augmentation of the presence of apoptotic cells in tamiCD4TR2 mice 2 weeks p.a. (**Fig. 9d**), correlating with the reduced number of T_n $CD4^+$ T cells at this time point. In order to investigate whether certain subpopulations of $CD4^+$ T cells upon removal of TR2 are more prone to apoptosis than the others, sorted T_{efm}, T_{reg} and T_n were cultured with 4-hydroxytamoxifen. Annexin-V staining after 20 and 40h of culture did not show a difference in apoptosis between TR2 deficient and respective control $CD4^+$ T cell population (data not shown).

The Bcl-2 protein, which is the member of pro-survival Bcl-2 family, was shown to be involved in the apoptosis of T_{reg} cells in CD4CreTR2 mouse model [108]. This anti-apoptoticprotein functions as a Bim antagonist. The expression of Bcl-2 in $CD4^+$ T cells isolated from tamiCD4TR2 mice 2 weeks p.a. was slightly decreased compared to control cells (**Fig. 9e**) and no difference in Bcl-2 expression was observed in $CD8^+$ T cells (**Fig. 9e**). The level of Bcl-2 was decreased in all $CD4^+$ T cells subpopulations isolated from tamiCD4TR2 mice (data not shown).

The cell death of $CD4^+$ T cells from tamiCD4TR2 mice was further analysed *in vitro* on sorted population. Cells were isolated from iCD4TR2 mice and control mice and susequently sorted into T_{reg}, T_n and T_{efm}. Cell death of the sorted population was more rapid and the incidence of apoptosis was much higher than T cell that were not isolated from total splenocytes (**Fig. 9f**). There was no difference in apoptosis between different $CD4^+$ T cell subsets. The experiment was repeated in a similar setup and sorted population were co-cultured together with feeder cells and with or without IL-7. This increased the survival of

$CD4^+$ T cells but there was no difference in incidence of apoptosis between TR2 deficient and control $CD4^+$ T cells (data not shown).

The observation that TGF-β-mediated suppression of proliferation affected only two T cell subpopulations prompted us to assess whether homeostatic regulation of these cell types comprises of a negative feedback loop involving autocrine production of TGF-β1. To test this hypothesis we analysed whether TGF-β1 was mainly produced by T_{reg} and T_{efm} cells. However, no major differences between sorted T cell subpopulations could be detected by qRT-PCR analysis (**Fig. 10**) in the wild type situation (CD4-$CreER^{t2}$/TGF-$\beta R^{f/+}$). TGF-β1 expression seems to be also independent of presence or absence of TR2 (**Fig. 10 a**). Thus, we can exclude an autocrine suppressive function of TGF-β1 for T_{reg} and T_{efm} cells through a transcriptional negative feedback loop. Interestingly, we observed however that the mRNA levels of the other component of the TGF-β receptor, TR1, were reduced specifically in the three T cell populations most-affected by TR2-deficiency (**Fig. 10 b**), which suggests a positive feedback loop for TR1 expression specifically in T_n, T_{efm}, and T_{reg} cells.

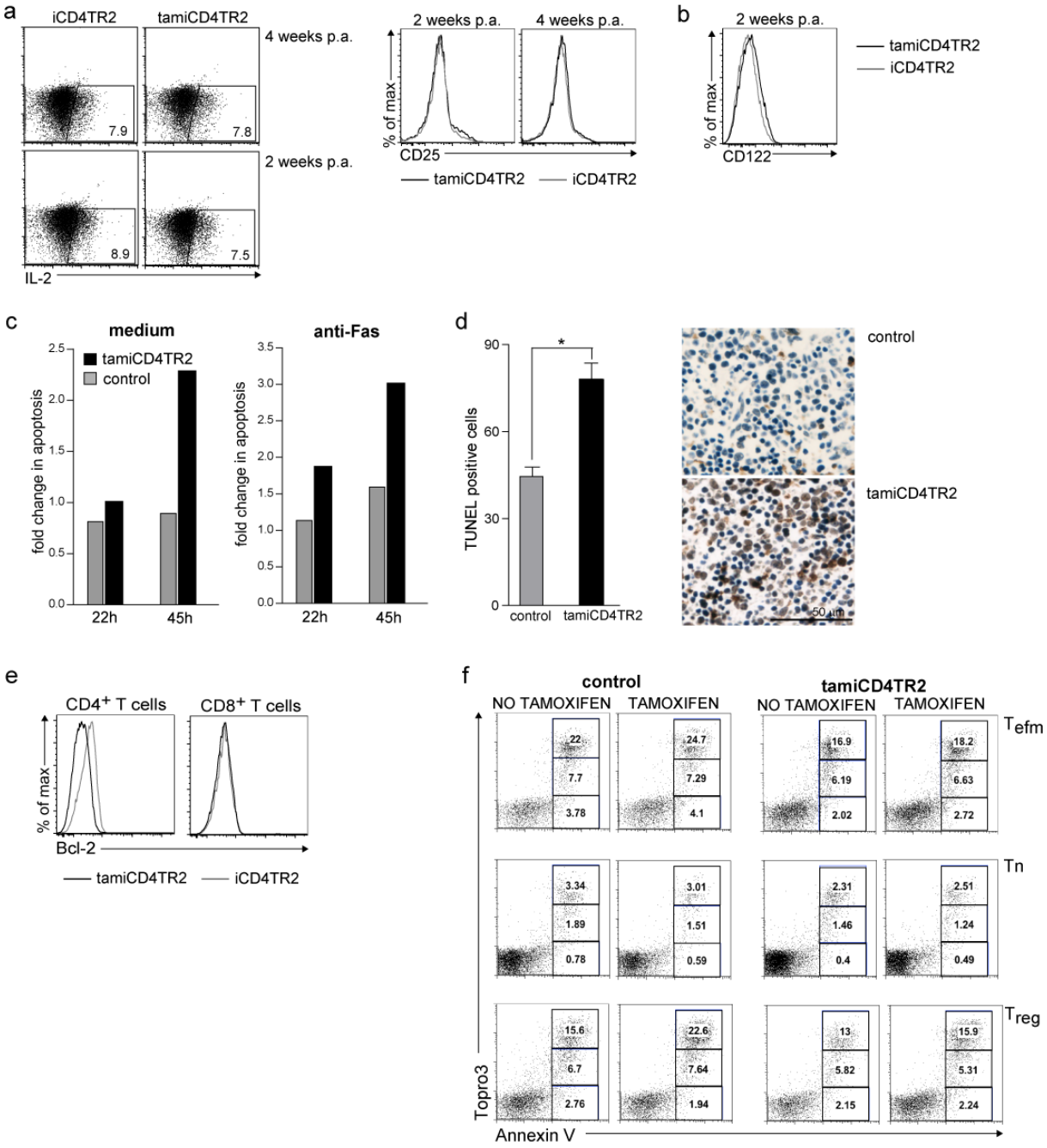


Fig. 9 Cytokine signaling and apoptosis upon removal of TR2.

(a) Flow cytometric analysis of IL-2 and CD25 expression by splenic $CD4^+$ T cells isolated from tamiCD4TR2 and control mice. (b) Flow cytometric analysis of CD122 expression by splenic $CD4^+$ T cells isolated from tamiCD4TR2 and control mice. Representative results of three independent experiments. (c) *In vitro* analysis of apoptosis induction. TamiCD4TR2 and control cells were cultured in AIM-V medium with or without tamoxifen. The ratio between AnnexinV positive $CD4^+$ T cells that were tamoxifen-treated versus untreated is indicated (mean, 3 mice per group). Representative of three independent experiments. (d) Representative micrographs of TUNEL-stained spleen sections from tamiCD4TR2 and control mice 2 weeks p.a. (40x) The size bar indicates 50 µm. Tunel positive cells were quantified by use of ImageJ software in five representative micrographs from each spleen. These data are representative of two independent experiments. (e) Flow cytometric analysis of Bcl-2 expression by splenic $CD4^+$ T cells isolated from tamiCD4TR2 and control mice. (f) *In vitro* analysis of apoptosis induction. Splenocytes from tamiCD4TR2 and control mice were sorted and cultured in AIM-V medium with or without tamoxifen for 72 hours

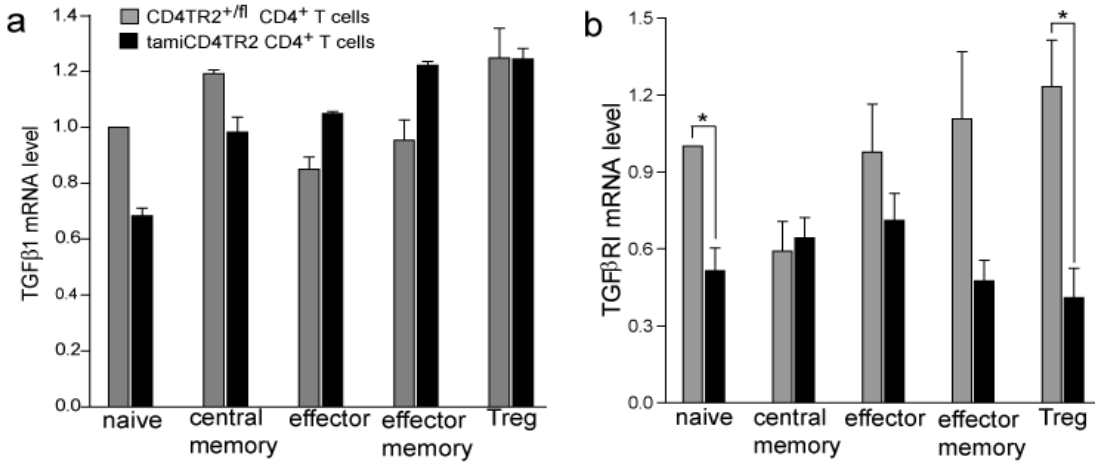


Fig. 10 Expression of TGF-βI and TGF-βRII

TGFRβ1 (a) and TGFβ1 (b) mRNA expression by the indicated CD4 T cell subpopulations FACS-sorted from spleen and LN (similar to Fig. 1) determined by quantitative RT-PCR at 2 weeks p.a.

The deregulation of T_{efm} and T_{reg} cells lacking TR2 is cell-intrinsic

To investigate whether the activation and proliferation of T_{reg} and T_{efm} cells were an autochthonous result of absence of TR2 or whether deficient cells were acting in a paracrine fashion, we generated mixed bone marrow chimeras (WT-CD45.1$^+$ and iCD4TR2 CD45.2$^+$ experimental or CD4-CreER^{t2}/TR2$^{f/+}$ CD45.2$^+$ control bone marrow) (**Fig. 11a**) that allowed us to determine whether the above-described phenotypic changes were restricted to the TR2-deficient population. 2 weeks p.a. the activated phenotype of CD4$^+$ T cells, defined by CD69, CD44, and CD62L was restricted to populations lacking the receptor (**Fig. 11b** and data not shown). Expansion of TR2-deficient (CD45.2$^+$) T_{efm} and T_{reg} cells was observed at 2 and 4 weeks p. a. (**Fig. 11 c,d,e**). The deficient T_n cell compartment was reduced while the mutant effector and central memory T cells were unchanged. These observations were confirmed by BrdU analysis 2 weeks p.a. showing increased proliferative activity in the TR2-deficient T_{reg} and T_{efm} cells but not in any other T cell population (**Fig. 11f**). Analysis of control chimeras showed no difference in percentages of cell types or incorporation of BrdU between the CD45.1$^+$ and CD45.2$^+$ populations. Thus, hyperactivation, increased proliferation of T_{efm} and

T_{reg} cells and the cell death-induced reduction of the T_n cell compartment are cell-intrinsic consequences of TR2 ablation.

TR2 deficient $CD4^+$ T cells were also co-transferred together with wt $CD45.1^+$ T cells into Rag-/- mice. Control animals obtained wt $CD45.1^+$ $CD4^+$ T cells and iCD4TR2 $CD45.2^+$ T cells. Mutant $CD4^+$ T cells expanded more than the co-transferred wt population. Also the $CD45.1^+$ T cells in experimental mice expanded to a higher extent than the both $CD4^+$ T cells populations in control animals. This suggests an additional extrinsic effect (**Fig. 12a**) that is IL-2 independent since neither IL-2 production nor CD25 expression were upregulated in experimental chimeras. 8 weeks after transfer only TR2-deficient $CD4^+$ T cells expanded much more much more than wt CD4 T cells co-transferred with transgenic cells and control $CD4^+$ T cells did (**Fig. 12a**). After 30 days post expansion TR2-deficient CD4 T cells produced significantly more IFNγ and together with co-transferred wt population less IL-2 (**Fig. 12b**). At the end of experiment lymphocytes from spleen and mesenteric lymph nodes were analysed. As for the earlier time point expression of IFNγ was upregulated and the production of IL-2 downregulated in $CD4^+$ TR2 deficient cells. The production of IL-17 by TR2-deficient $CD4^+$ T cells was significantly lower (**Fig. 12 c**).

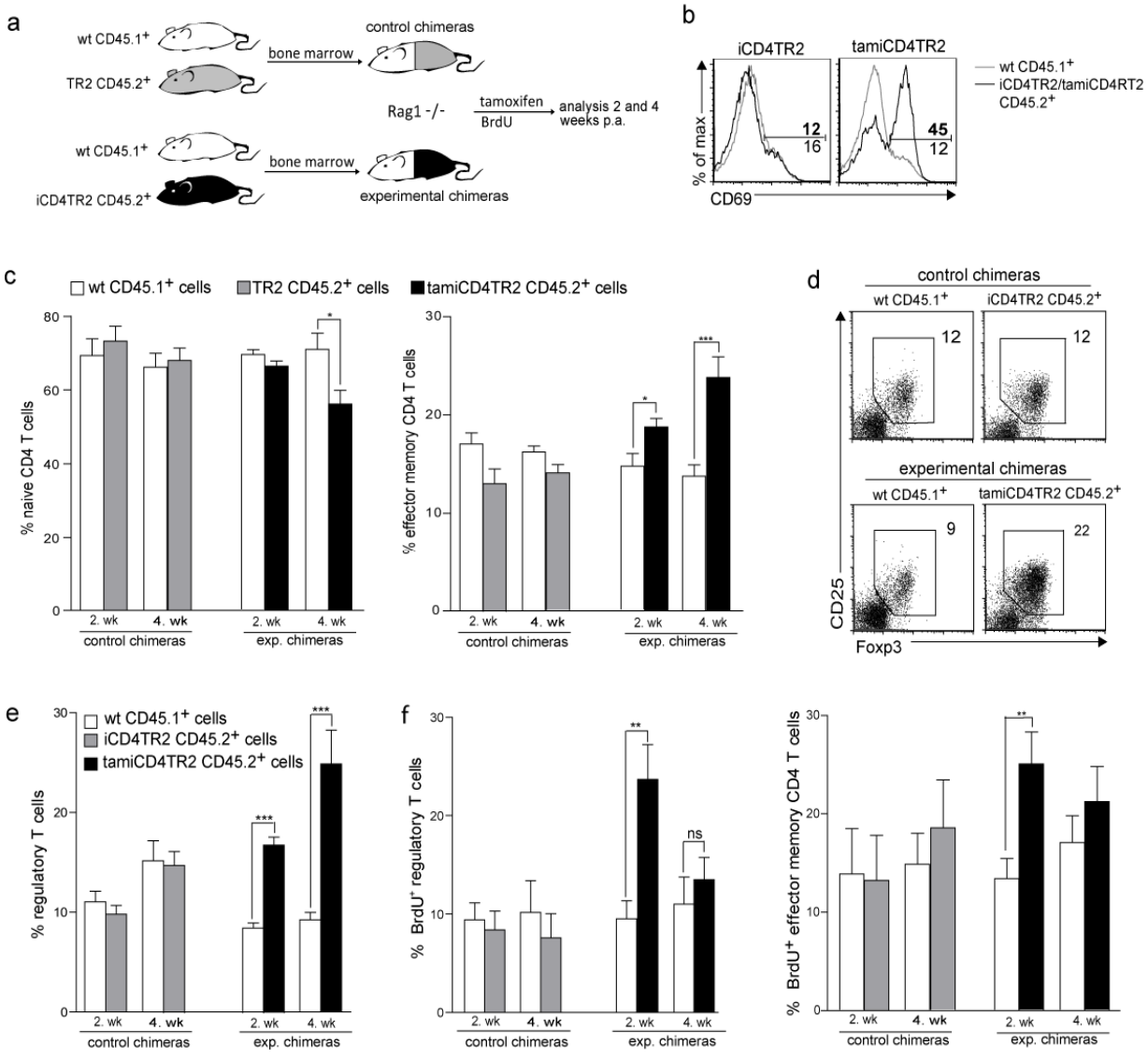


Fig. 11 The cellular changes are intrinsic to CD4$^+$ T cells lacking TR2

Rag1$^{-/-}$ mice were reconstituted with T cell-depleted bone marrow from wt CD45.1$^+$ and CD45.2$^+$ tamiCD4TR2 or iCD4TR2 in 1: 1 ratio and treated with tamoxifen for five consecutive days 5 weeks post reconstitution (a) Scheme of the experimental setup (b) Flow cytometric analysis of CD69 expression on CD4$^+$ splenic T cells isolated at 2 weeks p.a.. These data are representative results of three independent experiments. (c) The percentage of T_n and T_{efm} CD4$^+$ T cells, cells isolated from LN (mean ± SEM, 10 mice per group, analysed in three independent experiments). (d) Flow cytometric analysis of the expression of Foxp3 and CD25 by CD4$^+$ T cells isolated from LN at 2 weeks p.a (e) The percentage of T_{reg} cells within the lymph node CD4$^+$ T cells of the indicated CD45.1$^+$ or CD45.2$^+$ bone marrow-derived cells (mean ± SEM, 10 mice per group, analysed in three independent experiments). (f) The percentage of BrdU positive T_{efm} and T_{reg} cells isolated from LNs (mean ± SEM, 10 mice per group, analysed in three independent experiments).

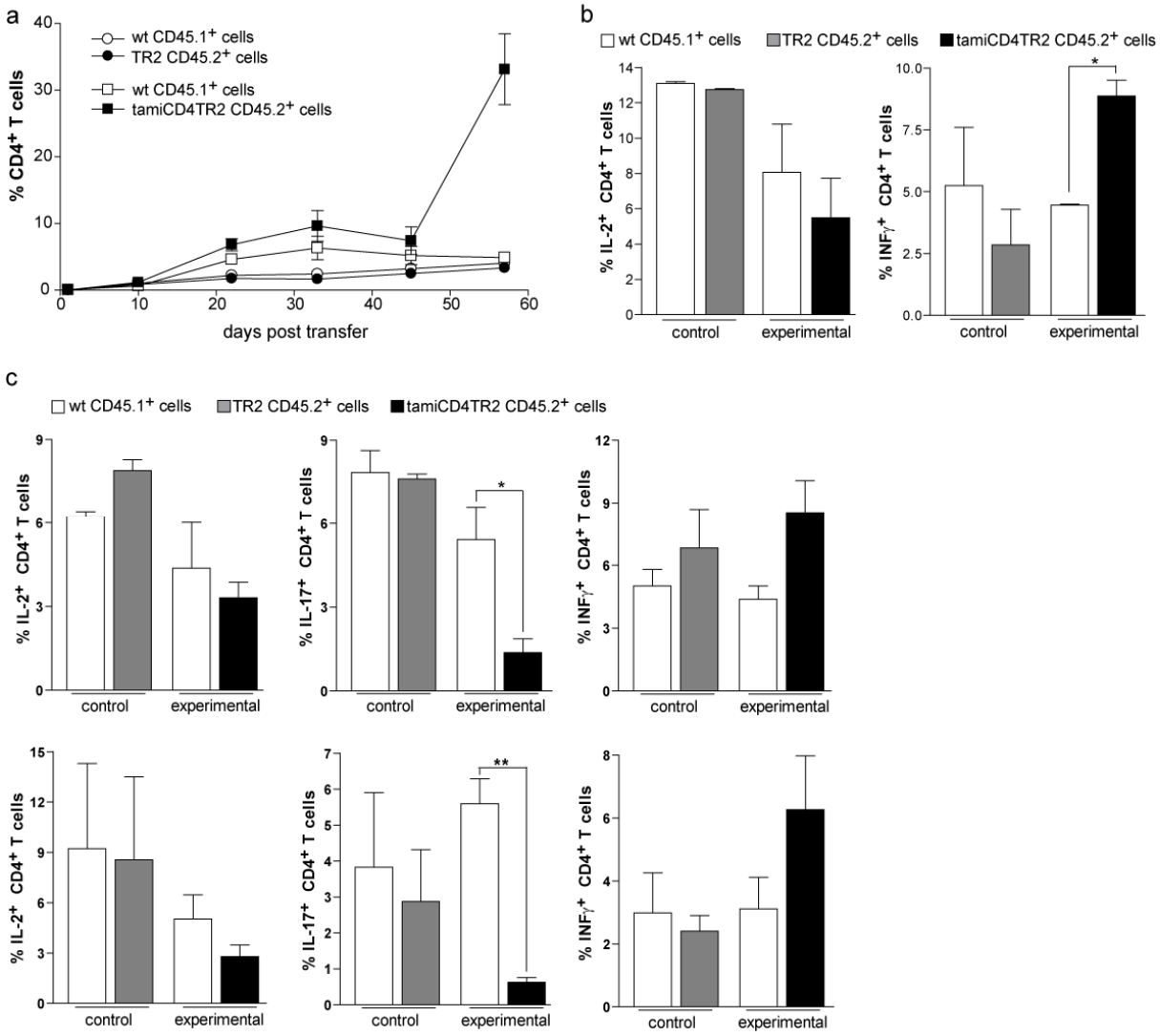


Fig. 12 Homeostatic expansion of TR2 deficient CD4$^+$ T cells in lymphopenic host

(a) Lymphocytes from tamiCD4TR2 or control iCD4TR2 mice were adoptively transferred (i.v) together with congenic CD45.1$^+$ wt T cells. The percentage of CD4 T cells in peripheral blood (mean ± SEM, 5 mice per group). These are representative results of two independent experiments. (b) The percentage of IL-2 and INFg CD4 T cells in the spleen after 30 days post transfer (c) The percentage of IL-2, IL-17 and INFg CD4 T cells in the spleen (upper row) and mesenteric lymph nodes (lower row) et the end of experiment.

TR2 expression by T_{reg} cells is irrelevant for their suppressive capacity *in vitro* and *in vivo*

Key features of T_{reg} cells are their expression of the transcription factor FoxP3 [98-99, 211] and of the surface proteins CTLA-4 and GITR (Tnfrsf18) [86, 212-213]. In the absence of TGF-β1 a reduction of FoxP3 expression levels was reported in peripheral T cells but not thymocytes [107]. A first assessment of the impact of removal of TR2 from T_{reg} cells showed FoxP3 levels to be unchanged in $CD4^+CD25^+$ cells between experimental and control animals at all analysed time points. CTLA-4 expression was found to be slightly upregulated while GITR levels remained the same in comparison to T_{reg} cells of control animals (**Fig. 13 a** and data not shown). We next examined *in vitro* whether TR2-deficient T_{reg} cells retained their ability to inhibit the proliferation of conventional wt T cells. Unexpectedly, we detected similar suppressive abilities by TR2-deficient and control T_{reg} cells (**Fig. 13 b,c**). We then tested whether TR2-deficient T_{reg} could also suppress mutant responder T cells, yet again with no change in T_{reg} cell suppression (**Fig. 13 d**).

Because of a seemingly opposing report [107] we performed an additional experiment and assessed suppression by mutant T_{reg} cells *in vivo* through a colitis inhibition assay. TR2-deficient or control T_{reg} cells were co-transferred with $CD4^+$ T_n cells into lymphopenic RAG1-deficient recipients. While transfer of T_n cells alone resulted in severe weight loss indicating colitis, the disease was similarly suppressed by co-transfer of T_{reg} cells from tamiCD4TR2 and control animals (**Fig. 13 e**). Furthermore, also colon histopathology did not reveal any difference between suppression by TR2-deficient and WT T_{reg} cells (**Fig. 13 f**). These results show that T_{reg} cells neither require TGF-β signalling for maintenance of Foxp3 expression nor for exertion of their suppressive abilities *in vitro* and *in vivo*.

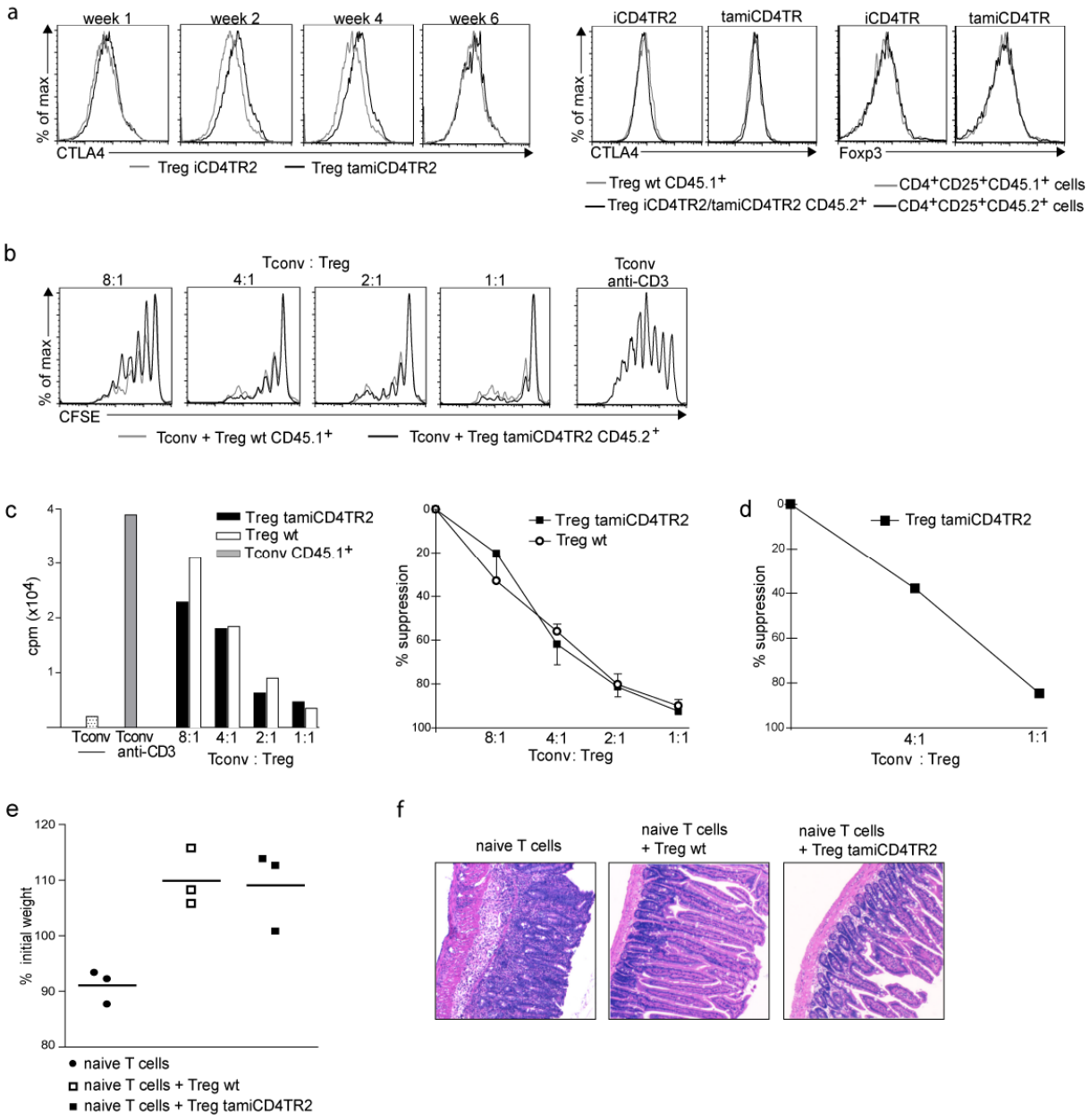


Fig. 13 T_{reg} cells lacking TR2 are functional

(a) Flow cytometric analysis of the expression of CTLA4 by T_{reg} and Foxp3 by $CD4^+CD25^+$ T cells isolated from LN of tamiCD4TR2 , iCD4TR2 mice and mixed bone marrow chimeras. For *in vitro* suppression assay sorted conventional $CD45.1^+$ T cells were labelled with CFSE (b) or pulsed with thymidine for the last 24h of culture (c), stimulated with anti-CD3 and cocultured with sorted iCD4RT2 T_{reg} cells or tamiCD4RT2 T_{reg} cells (isolated 14 days p.a.) at various ratios. Analysis was performed after 96h. (c) Left panel: proliferation assessed as uptake of $[^3H]$thymidine; right panel: percent suppression as mean ± SD (d) *In vitro* suppression assay: sorted conventional tamiCD4TR2 T cells were stimulated with anti-CD3 and cocultured with sorted tamiCD4RT2 T_{reg} cells at various ratios, pulsed with thymidine for last 24h. Analysis was performed after 96h (representative data of two independent experiment), (e) *In vivo* suppression assay: Development of colitis in $Rag1^{-/-}$ mice after adoptive transfer of conventional T cells alone or in combination with tamiCD4TR2 (mice treated for 5 days, cells isolated 1 week p.a.) T_{reg} cells or iCD4TR2 T_{reg} cells. Change in body weight after 8 weeks post transfer (mean, 3 mice per group, representative data of two independent experiments) (f) Representative micrographs of H&E-stained small intestine sections from colitis experiments as in (e) isolated from $Rag1^{-/-}$ 8 weeks after transfer of the indicated cells (20x).

The role of TGF-β signalling in skewing of CD4$^+$ T cells towards Th1, Th2 and Th17 cells

In mice expressing dnTGF-βRII under the CD4 promotor, an increased spontaneous differentiation of CD4$^+$ T cells into both Th1 and Th2 cells was reported [197]. However, CD4$^+$ T cells from mice with complete block of TGF-β signalling in T cells differentiated spontaneously exclusively into Th1 cells [50]. In the experiments performed with our inducible model we did not see changed differentiation of CD4$^+$ T cells into certain Th lineages even after thymectomy and long period tamoxifen treatment.

We wanted to test if there would be a difference in the expression of transcription factors or effector cytokines by TR2 deficient CD4 T cells *in vitro* in determined skewing conditions. To investigate the role of TGF-β signalling for T helper cell polarization, lymphocytes of tamiCD4TR2 mice were cultured under previously established conditions. Deletion of TR2 receptor from CD4$^+$ T cells was performed *in vivo* and cells isolated after 2 weeks p.a. were used for these experiments.

For the *in vitro* differentiation into T helper 1 cells IFNγ and IL-12 were added [158, 163] and cells that had differentiated into Th1 cells were identified by production of the signature cytokine IFNγ as well as up-regulation of the transcription factor T-bet. Surprisingly no difference between TR2 deficient CD4 T cells and control CD4 T cells in expression of T-bet or IFNγ production was found (**Fig.14 a**). Production of other cytokines that are not characteristic for Th1 cells was also analysed in this skewing condition. The percentage of GM-CSF producing CD4$^+$ T cells was significantly increased among TR2 deficient cells compared to control ones in both Th1 skewing condition and after stimulation with anti-CD3 and anti-CD28 antibodies (**Fig. 14 b**).

For the *in vitro* differentiation into T helper 2 cells, IL-4 and IL-2 were added to the cultures [171-172] and IFNγ and IL-12 signaling was inhibited by addition of the respective

antibodies. Analysis of $CD4^+$ T cells from tamiCD4TR2 and control TR2 animals cultured under Th2 skewing conditions revealed no differences in GATA-3 and IL-4 expression between TR2-deficient and control cells (**Fig. 15 a,b**). Nevertheless, it seems that only after three days of culture the percentage of GATA-3 positive cells was lower within TR2 deficient CD4 T cell suggesting possible differences in the kinetics of Th2 cells development (data not shown). CD4 T cells isolated from tamiCDTR2 mice produced significantly higher amounts of IL-5 under Th2 skewing conditions and strong stimulation of TCR than control T helper cells (**Fig. 15 c**). No difference was detected between cultured tamiCD4TR2 cells and control TR2 cells in IL-10 expression (**Fig. 15 c**).

For the *in vitro* generation of Th17 cells, TGF-β and IL-6 were added together with αIFNγ to induce expression of signature cytokines IL-17 and IL-21 [187-188]. In the absence of TR2 $CD4^+$ T cell produced significantly less IL-17 (**Fig. 16**). Instead the percentage of $CD4^+$ T cells producing IFNγ under these skewing conditions was higher among the tamiCD4TR2 T lymphocytes (data not shown).

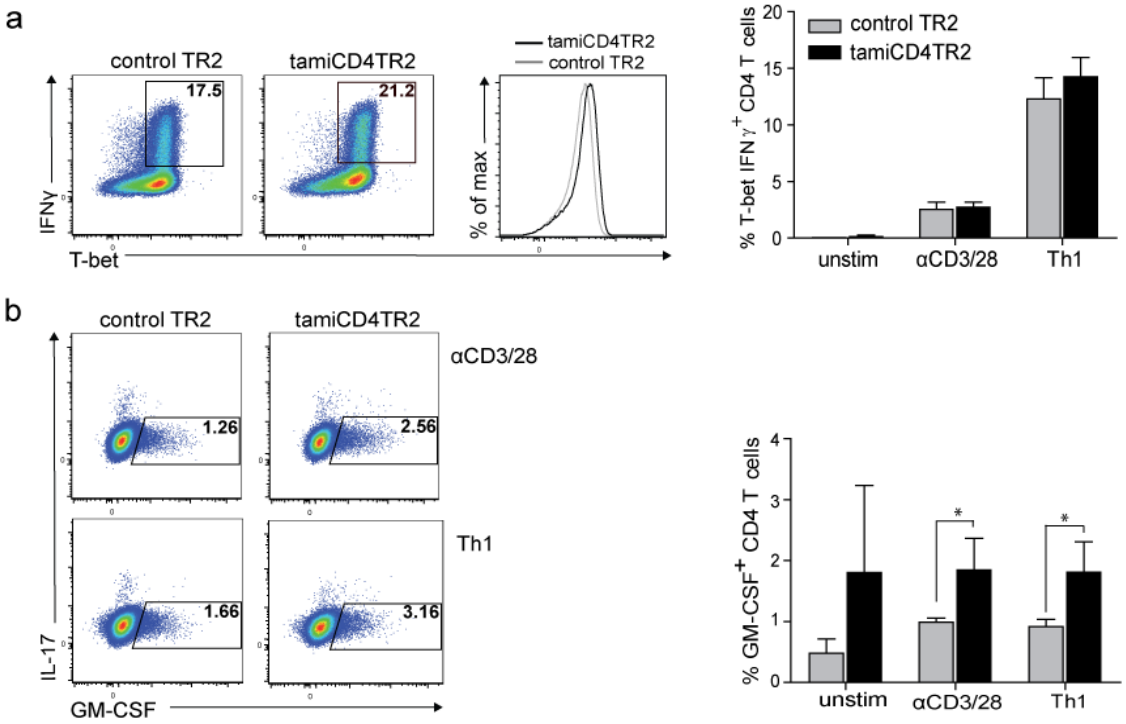


Fig. 14 *In vitro* Th1 skewing of tamiCD4TR2 and control CD4$^+$ T cells

tamiCD4TR2 and control TR2 cells were isolated 2 weeks post *in vivo* tamoxifen treatment and cultured in Th1 predisposing conditions and in αCD3/28 condition. (a): Flow cytometric analysis of IFNγ and T-bet expression by CD4$^+$ T cells cultured in Th1 skewing conditions for 3 days. These data are representative results of 2 independent experiments. The plot represents the percentage of IFNγ and T-bet postive cells within CD4$^+$ T cells under indicated conditions after 3 days (mean ± SEM, 5 mice per group analysed in 2 experiments). (b) Flow cytometric analysis of IL-17 and GM-CSF expression by CD4$^+$ T cells cultured in Th1 skewing conditions and αCD3/CD28 condition. The plot represents the percentage of GM-CSF positive cells within CD4$^+$ T cells under indicated conditions after 3 days (mean ± SEM, 5 mice per group analysed in 2 experiments)

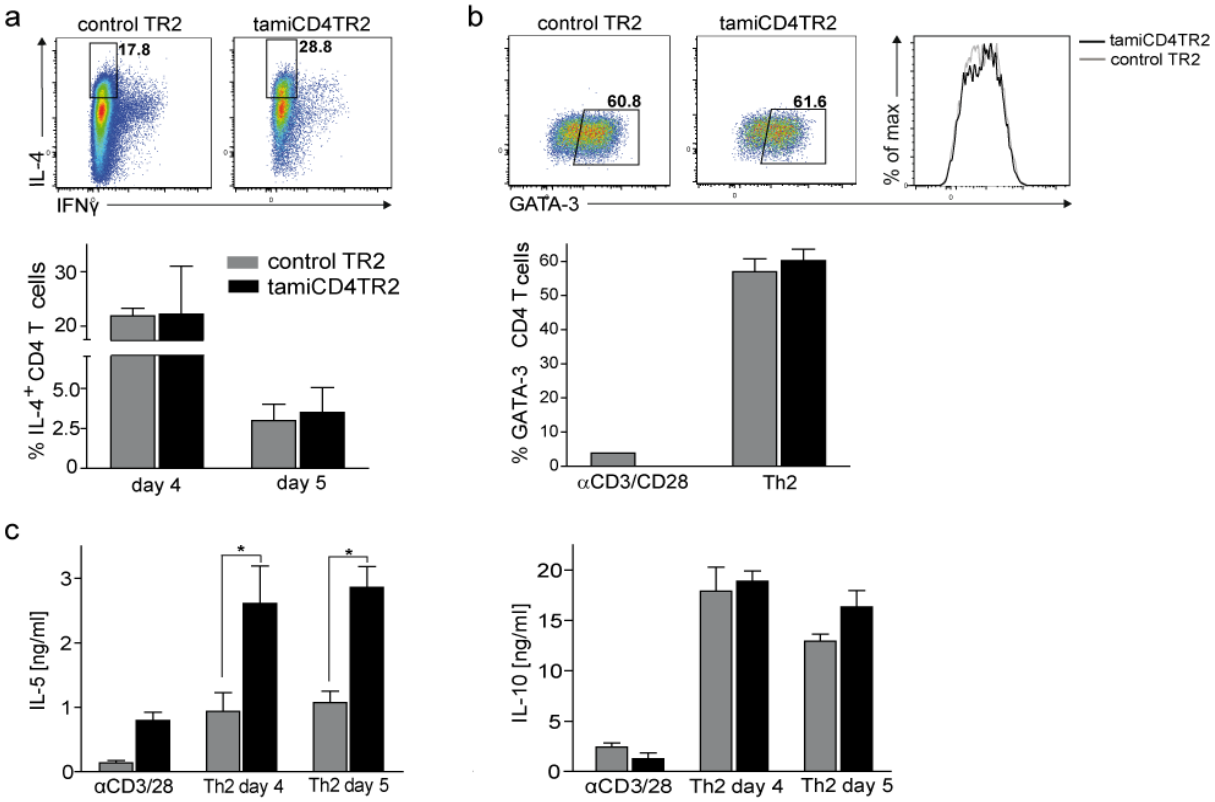


Fig. 15 *In vitro* Th2 skewing of tamiCD4TR2 and control CD4$^+$ T cells

tamiCD4TR2 and control TR2 cells were isolated 2 weeks post *in vivo* tamoxifen treatment and cultured in Th2 predisposing conditions and in αCD3/28 condition. (a) Flow cytometric analysis of IL-4 expression by CD4$^+$ T cells cultured under Th2 condition for 4 days. These are representative results of 3 animals analysed in one experiment. The plot represents the percentage of IL-4 positive CD4$^+$ T cells (mean ± SEM, 3 mice per group analysed in one experiment). B: Flow cytometric analysis of GATA-3 expression by CD4$^+$ T cells cultured under Th2 conditions for 5 days. The plots represent the percentage of GATA-3 positive CD4$^+$ T cells on day 5 (mean ± SEM, 5 mice per group analysed in 2 experiments for day 5; 3 mice analysed in one experiment for day 4). (c) The amount of IL-5 and IL-10 in the culture supernatants measured by ELISA (mean± SEM; 5 mice analysed in 2 independent experiments; * P<0.05).

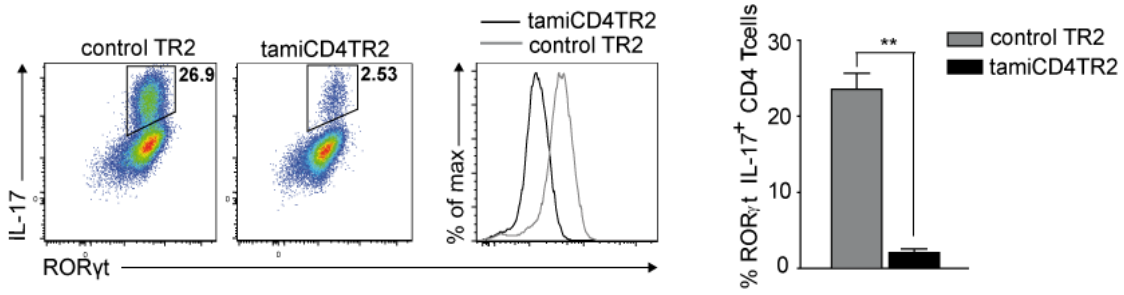


Fig. 16 *In vitro* Th17 skewing of tamiCD4TR2 and control CD4$^+$ T cells

tamiCD4TR2 and control TR2 cells were isolated 2 weeks post *in vivo* tamoxifen treatment and cultured in Th17 predisposing conditions for 4 days. Flow cytimetric analysis of IL-17 and RORγt expression by CD4$^+$ T cells. These are representative results of 3 animals per group analysed in one experiment. The plots represent IL-17 and RORγt positive or IL-17 positive T cells of 3 mice analysed in one experiment (mean± SEM).* P<0.05.

Altered T cell polarization *in vivo* in the absence of TGF–β signaling of CD4+ T cells

In steady-state condition, without immunization, the profile of cytokines produced by TR2 deficient $CD4^+$ T cells is only slightly different from that of control T helper cells. The amount of produced Th2 cytokines measured ex vivo without anti-CD3/CD28 stimulation is so low that it does not allow describing the differences in the ability of tamiCD4TR2 and control Th cells of their expression (data not shown). IFNγ production measured ex vivo is upregulated in TR2 deficient $CD4^+$ T cells but the difference is not as pronounced as described in a previously published model [49-50] (**Fig. 17**). Among the cytokines that were also upregulated is GM-CSF (**Fig. 17**). IL-17 is produced on a very low level and no difference in its expression could be observed. After stimulation of splenocytes with anti-CD3/CD28 the total amount of IFNγ and GM-CSF produced were not changed, whereas the level of IL-17 was significantly reduced after three days of culture (**Fig. 18**).

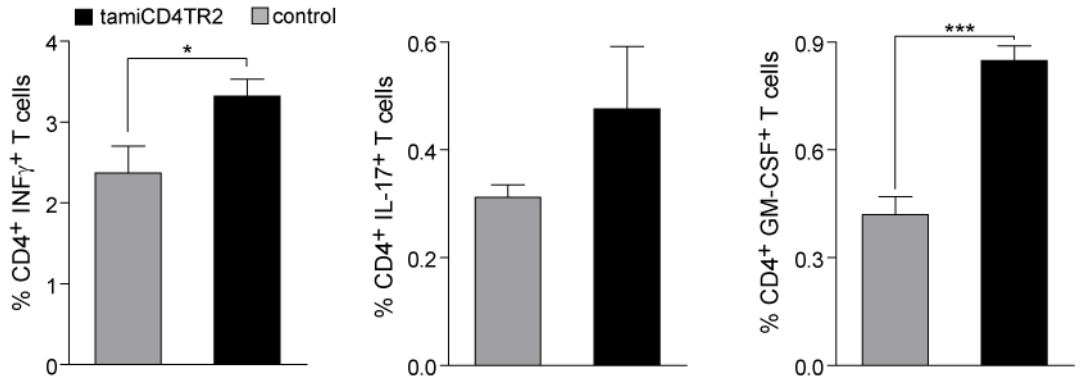


Fig. 17 Production of the cytokines in the steady state condition

Spleens from tamiCD4TR2 and control mice were isolated 2 weeks p.a.. Cells were stimulated with PMA/Ionomycin for 4h. The plots represents the percentage of $INF\gamma^+$, $IL\text{-}17^+$ and $GM\text{-}CSF^+$ $CD4^+$ T cells

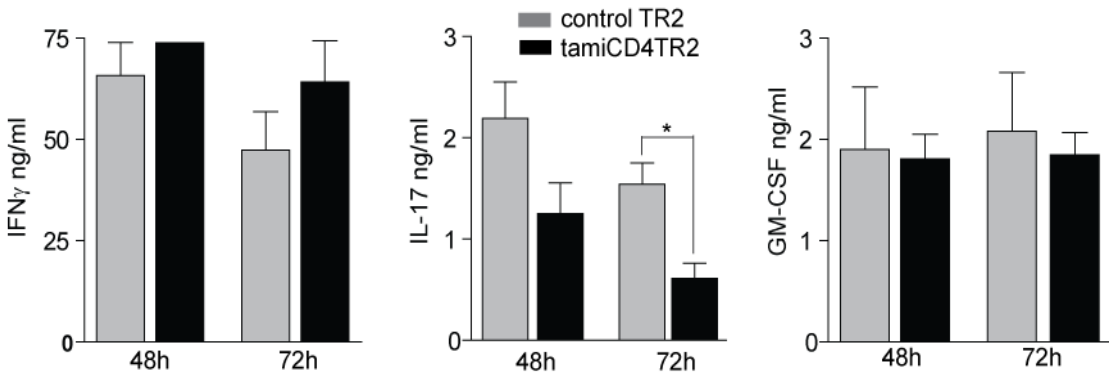


Fig. 18 Production of the cytokines after *in vitro* stimulation

Spleens from tamiCD4TR2 and control mice were isolated 2 weeks p.a.. Cells were stimulated with anti-CD3/CD28 for 48h and 72h. The plots represent the amount of cytokines in collected supernatants.

Due to the fact that the amounts of Th2 or Th17 cytokines produced by $CD4^+$ T cell in unimmunized mice are very low it is difficult to judge whether the mutated $CD4^+$ T cells have a different ability to produce these cytokines than wt CD4+ T cells do. We did not observe in steady-state conditions that $CD4^+$ T cells acquire a Th1 phenotype even after long tamoxifen treatment or treatment after thymectomy (data not shown). We therefore assessed the immune response in the presence and absence of TR2 on $CD4^+$ T cells by immunizing tamiCD4TR2 and control mice with the model antigen keyhole limpet hemocyanin (KLH). While we found that ten days post-vaccination the numbers of $CD4^+$ T cells in the draining lymph nodes were identical between tamiCD4TR2 and control mice the composition of $CD4^+$ T cell subset was changed. Similar to our observations in the steady-state the percentage of T_{reg} and T_{efm} cells were increased in the lymph nodes from experimental animals and $CD4^+$ T cells showed a more activated phenotype (data not shown). For analysis of proliferation and cytokine production we restimulated the lymphocytes *in vitro* with KLH and found that despite the increased numbers of T_{reg} cell the $CD4^+$ T cells from tamiCD4RT2 mice had increased proliferative abilities (**Fig. 18 a**). Also, TR2-deficient $CD4^+$ T cells showed decreased production of the cytokines IL-17, GM-CSF, IL-4 and IL-10 which define different Th cell

subsets, after 48 and 72h post restimulation (**Fig. 19 b**). Unexpectedly, secretion of IFNγ and IL-2 by T cells from tamiCD4RT2 mice was identical to the one from control T cells after 48 and 72 h, while the number of IFNγ-producing cells as measured by ELISOPT after 20h was higher for TR2-deficient T cells (**Fig. 19 b and c**).

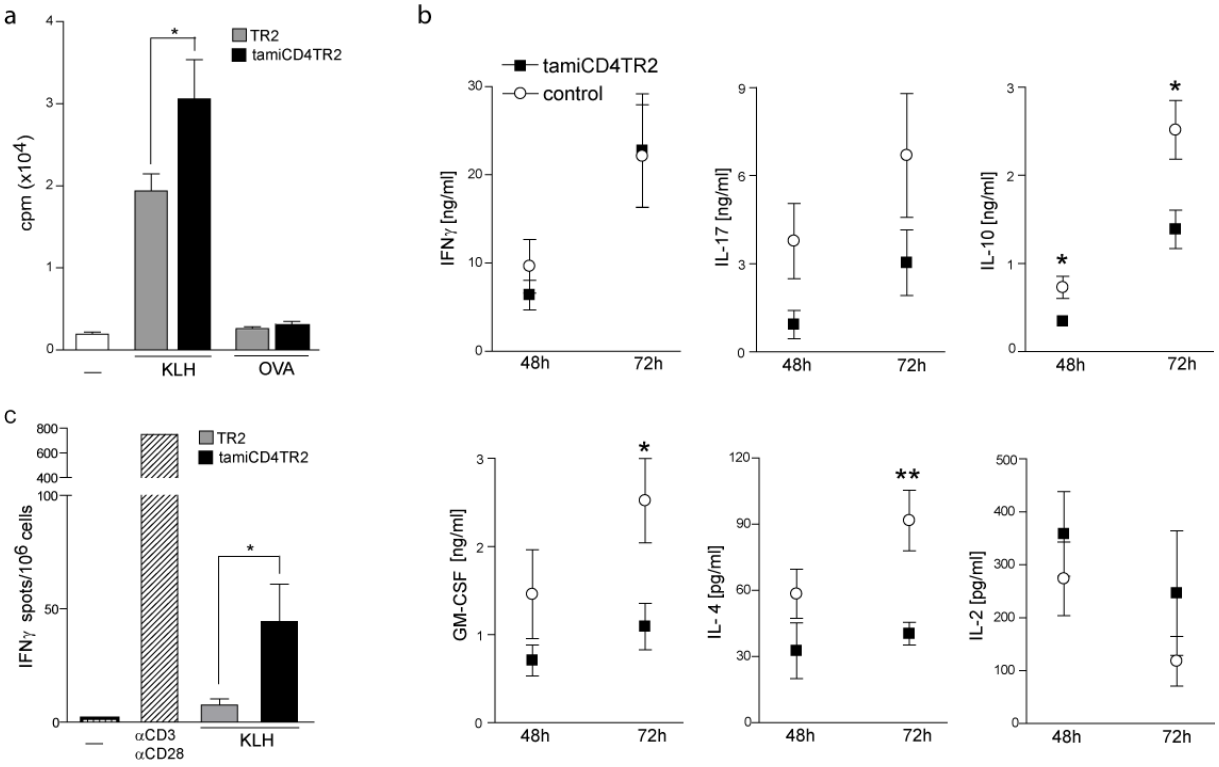


Fig. 19 Immune response after abrogation of TGF-β signalling

Mice were treated with tamoxifen for 5 consecutive days and immunized with KLH in CFA at day 14 p.a.. Spleen and draining LN were isolated at day 24 p.a. (a) Proliferation measured as thymidine incorporation after a 72 h restimulation with 50 μg/ml KLH, (representative data of two independent experiments, 6 mice per group) (b) Cytokine production measured by ELISA 48 and 72 h after restimulation with 50 μg/ml KLH or 50 μg/ml KLH and anti-CD28 (IL-4 and GM-CSF) (mean ± SEM, 12 mice per group analyzed in two independent experiments) (c) ELISPOT analyses of IFN-γ production by KLH-reactive lymphocytes restimulated for 20 h with 50 μg/ml KLH (mean ± SEM, 6 mice per group, representative data of two independent experiments)

Due to the fact that in tamiCD4TR2 mice the T_{reg} cells populations was significantly increased it is possible that the observed reduction in expression of certain cytokines are the result of a T_{reg} suppression rather than an intrinsic disability of TR2-deficient $CD4^+$ T cells to produce these cytokines. To overcome this caveat mixed bone marrow chimeric mice were generated in which $CD45.2^+$ iCD4TR2 or control bone marrow were mixed in a 1: 4 ratio with wt $CD45.1^+$ bone marrow. Unfortunately, the reconstitution in the spleen and lymph nodes after 5 weeks post transfer was 1:1 ($CD45.2^+$: $CD45.1^+$) even in wt control chimeras which does not allow us to exclude the extrinsic effect of regulatory T cells. After KLH immunization of these chimeras the observed phenotype in terms of T cell activation resembles the one of the immunized non-chimeric mice (data not shown). To examine the cytokine production cells were restimulated with KLH and stained and analysed after 6 h. The data for cytokine expression were normalised to the percentages of effector cells within the certain subpopulation. There was no significant difference in the production of IFNγ between TR2-deficient and wt $CD4^+$ T cells. Expression of IL-17 was significantly decreased in mutated compartment whereas GM-CSF production was increased, which confirm the data from previous *in vitro* experiments (**Fig. 20**)

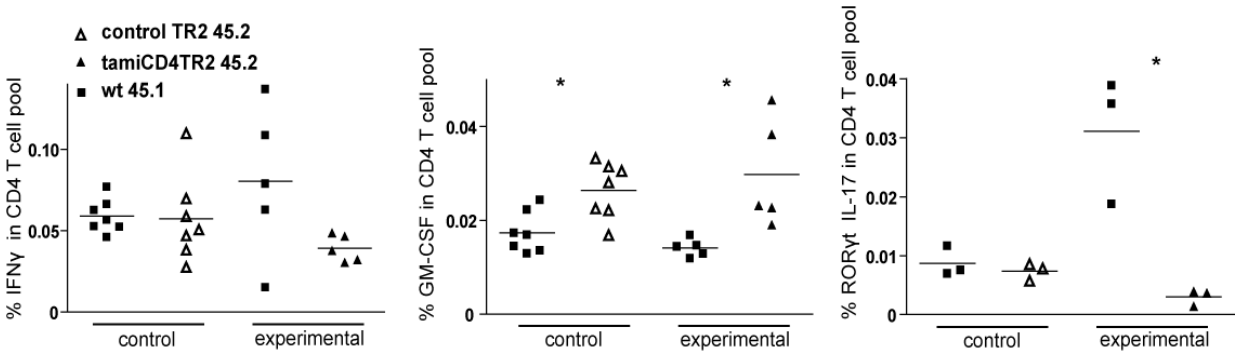


Fig. 20 Immune response in mixed bone marrow chimeras after abrogation of TGF-β signalling

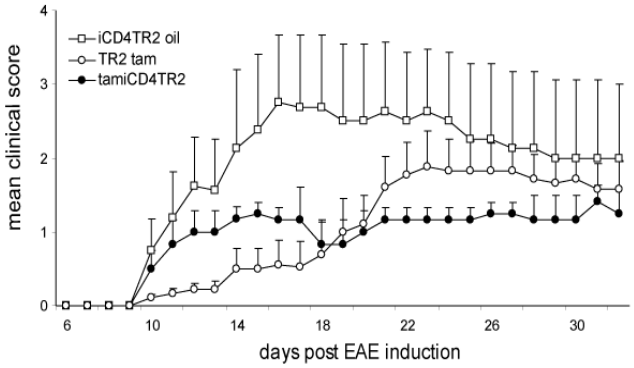
Percentages of cytokine producing cells were first normalized to their parent $CD45.1^+$ and $CD45.2^+$ population and than divided by the respective normalized number of effector and effector memory $CD4^+$ T cells. The plots represents the percentages of IFNγ, GM-CSF and IL-17 positive $CD4^+$ T cells among effector T cells in the total $CD4^+$ T cell pool of the mouse (mean, 3 to 7 mice per group analysed in one experiment).

Analysis of the role of TGF-β signaling in CD4 T cells in experimental autoimmune encephalomyelitis (EAE)

The role of TGF-β in EAE was studied in many mouse models including T cell-specific knockouts of TR2 or TGF-β1 [95, 187]. In both models mice were less susceptible to EAE which was only correlated with the notion that $CD4^+$ T cells in these mice produced less IL-17.

In our tamiCD4TR2 mice upon immunization with KLH we observed differences in production of IL-17, GM-CSF and some Th2 cytokines by TR-deficient $CD4^+$ T cells. That is why we wanted to investigate the role of TGF-β signaling in Th cells for the development and severity of EAE in tamiCD4TR2 mice. The same setup as for KLH immunization was applied. TamiCD4TR2 and control mice were immunized with MOG/CFA 2 weeks pa.. There was no difference in the day of EAE onset but the severity of disease was ameliorated (**Fig. 21 a, b**). However, tamoxifen-treated control mice suffered from less severe EAE than oil-treated controls (**Fig. 21 a, b**) which suggest tamoxifen side-effect affecting EAE severity.

a



b

	iCD4TR2 oil	TR2 tam	tamiCD4TR2
incidence (%)	100	100	100
mean day of onset	10	10.7	10
mean max severity	2.69	2.05	1.25

Fig. 21 EAE course after abrogation of TGF-β signalling in CD4$^+$ T cells.

tamiCD4TR2 and control mice tamoxifen or oil treated were immunized with MOG/CFA 2 weeks p.a. (a) disease course. (b) EAE disease incidence, mean time of disease onset and mean maximum clinical score of diseased mice.

Therefore, another experimental setup was tested to investigate the effect of TR2 ablation on the development of EAE. The general approach to exclude a tamoxifen side-effect on EAE was to not perform tamoxifen treatment and MOG-immunization in the same mouse. To achieve this, lymphocytes from tamoxifen-treated CD4TR2 2d2 [214] and control mice were transferred into RAG1$^{-/-}$ recipients, followed by EAE induction after transfer. The percentage of naïve CD4$^+$ T cells of 2d2 tamiCD4TR2 was reduced compared to 2d2 control TR2 animals, as described for tamiCD4TR2 animals. The percentage of regulatory T cells was only slightly increased in 2d2 tamiCD4TR2 compared to 2d2 control TR2 animals (**Fig. 22 a**). All recipients were actively immunized with MOG/CFA four hours after cell transfer. All animals in both groups developed severe EAE. There was no difference in EAE onset or the severity of the disease between experimental and control mice (**Fig. 22 b,c**)

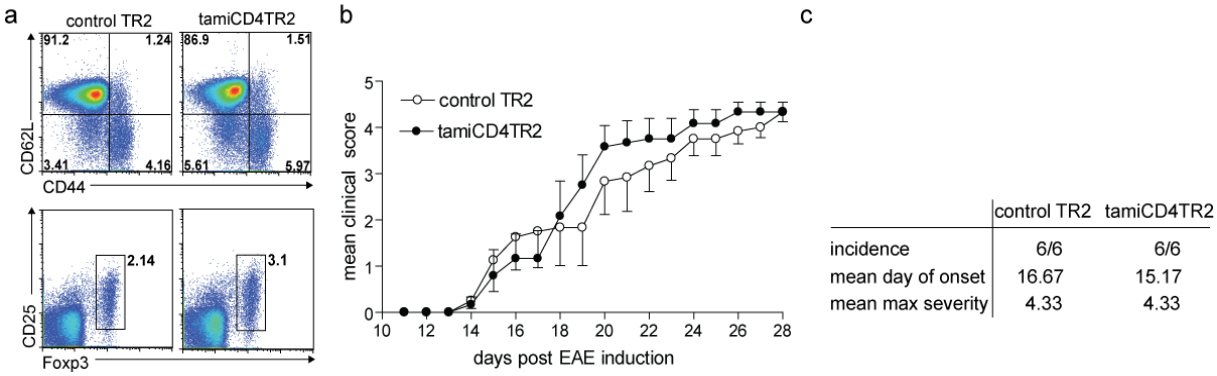


Fig. 22 Active EAE induction after transfer of tamiCD4TR42d2 and control cells.

Donor CD4TR2 2d2 and control TR2 2d2 mice were treated with tamoxifen for 5 consecutive days. Cells for transfer were isolated 7 days after first day of tamoxifen treatment. Recipient RAG1-/- animals received 20 million cells. Recipient mice were immunized with MOG/CFA 4 hours after cell transfer. (a) Flow cytometric analysis of CD44, CD62L, CD25 and Foxp3 expression by transferred CD4+ T cells. (b) The graph represents the mean clinical EAE course over time post immunization (mean± SEM, 6 animals per group were analysed in one experiment). By day 28 p.i. all animals were dead. (c) EAE disease incidence, mean time of disease onset and mean maximum clinical score of diseased mice.

DISCUSSION

Tamoxifen-inducible Cre-ER^{t2} mouse models

Several strategies were developed to achieve more precise spatial and temporal control of gene expression or modification in the mouse. The first available inducible Cre system was the Mx-Cre system [215] in which the Cre activity is regulated on a transcriptional level. The Cre recombinase is expressed under the control of an interferon-inducible promoter. The most popular nowadays is the tamoxifen inducible Cre-ER^{t2} system. It consists of the Cre recombinase which is fused to mutated human estrogen receptor (ER) ligand-binging domain [216-218]. The mutated ER domain binds the estrogen antagonist 4-hydroxytamoxifen but not the natural ligand β–estradiol. Without ligand Cre-ER^{t2} stays in cytoplasm bound to heat shock protein (HSP90). After binding of the ligand to ER domain conformational changes occur and HSP90 dissociates from the fusion protein. Cre-ER^{t2} is trans-located to the nucleus where the Cre recombinase can bind to and recombine respective loxP sites. Several mice that express Cre-ERt2 under tissue-specific promoters were generated so far. In 2001 the tamoxifen inducible adipocytes specific mouse was reported in which Cre-ER^{t2} was expressed under the control of the adipocyte fatty acid binding protein (aP2) [219]. The Cre activity in this model was specific to adipocytes reaching 80% of recombination based on western blot analysis. The group of Hayashi reported a line that exhibited widespread expression of Cre-ER [220] under a chimeric promoter consisting of the cytomegalovirus immediate-early enhancer and chicken-actin promoter/enhancer (CAGG). After tamoxifen application recombination was shown in embryos reaching more than 50%. Other tissue specific inducible mouse lines were generated in 2003 by the group of U. Suter [221]. The reported mice express Cre-ER^{t2} under the control of two different promoters that are specifically active in oligodendrocytes and/or Schwann cells. The Cre specificity and activity was tested in adult animals on the basis of β-galactosidase expression. The percentage of recombination varies in

different parts of the brain from 75 to 90%. Another example of a tamoxifen-inducible mouse strain is the vil-Cre-ERt2 mouse in which Cre is expressed under the epithelium specific villin promoter [222]. No quantitative analysis was performed with this strain but β-galactosidase activity was specific for the digestive tract epithelium.

A transgenic mouse expressing Cre-ER^{t2} in the ventricular zone of the developing nervous system under the control of the nestin promoter and enhancer (Nes-CreER^{t2}) was published in 2006 [223]. After crossing this mouse to the EGFP reporter strain the Cre-mediated recombination upon tamoxifen treatment was detected in 10-50 % of neuronal progenitors. A similar Cre activity was obtained in a model where CRe-ERt2 was expressed under a promoter specific for dorsal root ganglion (DRG) neurons [224].

So far there is only one tamoxifen -inducible Cre strain specific for hematopoietic cells reported - the Em/PSV40 Cre–EBD mouse [225]. In this case the immunoglobulin heavy chain enhancer (Eμ) was combined with the SV40 early minimal promoter to direct transgene expression exclusively to B lymphocytes. The maximal recombination that was observed in this model reached 65%. As Cre was not expressed in all IgM+ cells, the 65% maximum of observed recombination reflects >80% recombination in Cre-expressing cells.

Our CD4Cre-ER^{t2} mouse is the first tamoxifen inducible T cell specific mouse model. The Cre-ER^{t2} expression is driven by the CD4 promoter which is active from the DP stage on in T cell development. To judge Cre specificity and activity we crossed the mice to two different reporter mouse strains: RAGE-EGFP [207] and ROSA-EYFP [204]. After tamoxifen treatment of adult animals we achieved 70 to 80% of recombination solely in CD4$^+$ T cells which in comparison to other inducible mice is very efficient. After the treatment of adult mice we did not observe any Cre activity in CD8$^+$ T cells or other cells types that do not express CD4. As expected some recombination was detected in CD4-expressing splenic DC subsets and NKp46$^+$ LTis. There was also no EGFP$^+$ or EYFP$^+$ CD4$^+$ T cells present before tamoxifen application which means that the construct is very tight. By crossing CD4-CreER^{t2}

mouse with a Flp deleter mouse [208], Flp-mediated recombination was used to excise the neomycin selection cassette. Astonishingly, the percentage of recombination after removing neomycin was much lower than with the neomycin cassette. The influence of the neomycin selection cassette on the expression level was reported before [209]. Therefore for further experiments only the strain with neomycin cassette was used.

Different ways and doses of tamoxifen application were tested in our strain. We did not observe any obvious changes in the behavior, feeding, or appearance of the mice even after the long term, 60 days treatment. Tamoxifen and 4-OH tamoxifen were also tested for the *in vitro* recombination of target alleles in $CD4^+$ T cells. In contrast to some reports we did not observe an increased in the cell death of activated cells after co-culture of splenocytes with these ER^{t2} ligands.

After testing the Cre specificity and efficiency in CD4Cre-ER^{t2} mouse crossed to reporter strains, our new mouse was crossed to TGFβ-RII strain [210]. Due to technical problems with TR2 surface staining it is difficult to exactly calculate the percentage of $CD4^+$ T cells that have deleted the receptor after tamoxifen treatment. We observed a reduction from 80% to 10% of $CD4^+$ T cells expressing TR2 which means that the percent of recombination with this target was similar to one achieved with the RAGE EGFP reporter strain. In addition to the TR2 surface staining the analysis on mRNA level was performed. In tamiCD4TR2 animals the expression level of TR2 was reduced to an average 5% of control TR2 animals, which is very efficient deletion as for an inducible system. We also performed functional assays *in vitro* to show that $CD4^+$ T cells from tamiCD4TR2 mice do not respond to TGF-β signaling. TGF-β together with IL- 6 is known to induce the expression of IL-17 and RORγ [185]. In contrast to control $CD4^+$ T cells, tami$CD4^+$ T cells did not produce any IL-17 upon stimulation with IL-6 and TGF-β which confirms the efficient abrogation of TGF-β signaling in our model.

The role of TGF-β in central tolerance

The pleiotropic nature of the TGF-β family members has made it extremely challenging to unravel the role of these messengers within individual functional systems. The analysis of general and T cell-specific gene-deficiencies for TGF-β1 and its signalling pathways have invariably revealed severe autoimmune phenotypes, some with rapid death shortly after birth [49-50, 95, 120-121, 197, 210]. These observations resulted in the notion of TGF-β being required for maintenance or establishment of T cell tolerance. Also, T cell-restricted double deficiency of Smad 2 and Smad 3, members of the canonical TGF-β signalling cascade, but not their single deficiencies, led to severe inflammation in various organs followed by death [202, 226-227]. Yet, because gene ablation in all these systems took place or was present during thymic development it cannot be excluded that the observed immune deregulation was a consequence of T cell development in the absence of TGF-β signals. To overcome this caveat and analyse the role of TGF-β signaling only in post-thymic $CD4^+$ T cell we treated adult iCD4TR2 animals with tamoxifen. Since there was no change in expression of TR2 in $CD8^+$ T cells we can assume that recombination took place only in the periphery not in the DP stage of thymic development. We did not see any changes in thymus in terms of cellularity, $CD4^+$ to $CD8^+$ T cell ratio or expression of the markers like CD5, CD24 and CD69. Deletion of TGF-βRII in T cells in one of the studies resulted in decreased $TCR^{hi}CD8^+$ SP T cells compartment and accumulation of DP thymocytes [49]. In contrast to other reports the nT_{reg} population in the thymus was not increased [49, 109].

TGF-β was reported to be involved in the development of the iNKT (invariant NKT cells), a subclass of NKT cells ([49, 51]. In our studies we did not observe a difference in the number of NKT cells. Recent studies showed the role of TGF-β in the development of yet another subpopulation of T cells: $CD8\alpha\alpha^+$ intestinal intraepithelial lymphocytes (IELs) [228]. The development pathway of this population was under debate but now they are considered to be

of thymic origin. TGF-β was found to inhibit the apoptosis of the precursors of CD8αα$^+$ IELs and also to induce and maintain CD8α expression in these cells. Still is not clear if diminished number of CD8αα IELs in the intestine in CD4Cre TGF-βRI mice can contribute to development of autoimmunity. The authors showed as well that TGF-β promote expression of CD8α in thymocytes by diminishing Th-POK transcription factor.

It is tempting to speculate that negative selection in the thymus depends on TGF-β signalling, leading to emigration of autoreactive clones in its absence. Indeed, a recent study found exaggerated negative selection in absence of TR2 [108], an outcome that is in line with decreased but not increased autoimmunity. We did not study in our model in detail thymic development by crossing iCD4TR2 mice to RIPmOVA mice, so we cannot exclude that in our model negative selection is affected to some extent.

We are currently investigating if the ablation of TGF-β signaling from CD4$^+$ T cells in our model during the thymic development is causing autoimmune inflammation. To answer this question we generated bone marrow chimeras with iCD4TR2 and control bone marrow. The recipient mice were treated with tamoxifen before reconstitution and after such that the CD4$^+$ T cells had abrogated TGF-β signaling already from the DP stage of development. In the very first experiment the experimental chimeras develop disease four weeks post reconstitution. The changes in CD4$^+$ T cell compartment, infiltration of the leukocytes to the organs and presence of autoantibodies in these mice are currently investigated.

To summaries, so far in our tamiCD4TR2 mouse we did not observe any changes in thymic development of any CD4$^+$ T cell subset after treatment of tamoxifen adult animals.

The role of TGF-β in peripheral tolerance and control of autoimmunity

Despite efficient TR2 gene ablation in mature CD4$^+$ T cells in tamiCD4TR2 mice we did not observe any clinical or immunological manifestations of autoimmunity in our model, in contrast to all other models of modified TGF-β signalling in T lymphocytes [49-50, 109-

110, 120, 210]. Even when we employed more stringent experimental setups, including thymectomy and long-term tamoxifen application for 60 days, no indications of tolerance loss like the presence of autoantibodies could be found. We are confident that the ~10–20% unmodified cells remaining in our model are not the cause for the observed lack of autoimmunity because two groups have shown independently that chimeras with 50% CD4-cre/TR2$^{f/f}$ and WT bone marrow still develop the lethal autoimmune syndrome[49 , 50]. Even upon adoptive transfer of TR2-deficient cells into WT recipients vigorous proliferation of these cells within the otherwise normal environment was observed [49]. Since we did not detect a depletion of the T_{reg} cell compartment similar to the other systems [49-50, 107] the lack of autoimmunity could have been attributed to suppression by these cells. Yet, again it has been shown by complete repopulation of the T_{reg} cell compartment through transfer of WT T_{reg} cells into 2 days old CD4-cre/TR2$^{f/f}$ mice that T_{reg} cells cannot prevent or ameliorate the autoimmune syndrome [49]. Thus, we are confident that the incomplete gene deletion does not account for the divergent phenotype between the constitutive and our inducible model. Autoimmunity has, however, been observed in another setup of peripheral TR2 ablation in which *in vitro* TAT-Cre treatment was followed by adoptive transfer into RAG-deficient mice [50], thus seemingly contradicting our data. Yet, activation and expansion of the transferred T cells followed by development of autoimmunity may in this case be the result of naïve T cells being transferred into lymphopenic hosts [229]. Two explanations could account for the absence of autoimmunity after peripheral TR2 ablation, namely unmodified T cell development or restriction of the gene modification to CD4$^+$ T cells. As mentioned before, it is very unlikely that in our model T cell development and selection processes in the thymus are affected.

One report placed the pathogenic activity of TR2-deficient T cells in unconventional NK T cells without αGalCer reactivity [50]. We could find neither an increase in such NK1.1$^+$ T cells nor did we observe any other obvious phenotypes within the NK T cell population,

supporting the notion that these cells are involved in the auto-inflammatory response of CD4-cre/TR2$^{f/f}$ animals. However, other studies using cell ablation or MHC class I and II deficiencies indicated that T cells responsible for the autoimmune phenotype are found in both the CD4$^+$ and CD8$^+$ compartments [50, 230-231]. Hence, restriction of TR2 ablation to CD4$^+$ T cells without affecting CD8$^+$ T cells could only explain amelioration but not a complete absence of autoimmunity.

Changes in peripheral CD4$^+$ T cell subsets upon abrogation of TGF-β signalling

Even though we did not observe the anticipated autoimmune phenotype when TR2 was absent only from peripheral CD4$^+$ T cells, we detected severe abnormalities within the CD4$^+$ T cell compartment. Most prominently, T_{efm} and T_{reg} cell numbers and percentages were strongly increased in the absence of TR2, with both populations being activated (CD69$^+$) and cycling (BrdU$^+$). The expansion and proliferation of T cells has been reported also in the T cell-specific constitutive models of absent TGF-β signalling, albeit to a much stronger extent than in our model [49-50, 110]. This more pronounced deregulation may actually contribute to or even allow the development of the autoinflammation in these models. In one DN-TR2 strain, however, only CD8$^+$ T cells showed loss of cell cycle control[120], possibly an effect of expression variegation. In studies of TR2 as well as TGF-β1 deficiency the hyperproliferative phenotype of T cells has been attributed to increased CD122 expression and thus increased responsiveness to IL-2 and IL-15, cytokines involved in T cell homeostasis [49, 95]. In contrast to these observations we do not see in our model any increase in IL-2 production and only a slight increase in CD122 expression (IL2Rβ chain), making it unlikely that this pathway is involved in the observed loss of cell cycle control in T_{efm} and T_{reg} cells. As described before[49-50] also in our model the introduction of a TCR transgene (MOG35-55 specific 2D2 transgene [214]) resulted in ameliorated proliferation (data not shown)

suggesting that a specific TCR signal through recognition of self or environmentally present antigens is required for induction of the observed proliferation.

Similar to what the others reported we observed an increase in apoptosis both *in vitro* and *in vivo* after abrogation of TGF-β signalling in $CD4^+$ T cells [49, 108]. The level of proapoptotic protein Bcl-2 was downregulated similar to what was described in CD4Cre-TR2 mice. At this stage we cannot show which of the $CD4^+$ T cells subsets is the most prone to apoptosis. The Bcl2 level in was equally downregulated in regulatory T cells, memory or naïve cells. We observed a drop in the numbers of naïve T cells in the spleen and lymph nodes after two and four weeks p.a., which could suggested that these T cells were dying faster. However, it is also possible that naïve T cells acquire first effector phenotype and then undergo apoptosis.

Upon co-transfer of tamiCD4TR2 $CD45.2^+$ T with congenic wt cells into a lymphopenic host, transgenic $CD4^+$ T cells expanded faster and to a higher extent than wt cells. The expansion of both co-transferred population was higher than the control $CD45.2^+$ population and congenic cells. We investigated possible pathways involved in T cell proliferation. The IL-2 production was decreased by both more expanding populations. One of the reasons for this could be that these $CD4^+$ T cells deviated from memory to an exhausted phenotype which was reported already in chronic infectious models [232].

As reported before, $CD4^+$ T cells upon abrogation of TGF-β signalling also in our model acquire memory phenotype and become hyperproliferative but it is unlikely that this is dependent on their increased responsiveness to IL-2 and IL-15.

Regulatory T cells

In contrast to T_{efm} cells which seem to have increased population sizes in both the constitutive and the induced models of TR deficiency, T_{reg} cells exhibit different outcomes dependent on when TGF-β signalling is abrogated. Previously, the almost complete absence of peripheral T_{reg} cells in CD4-Cre/$TR2^{f/f}$ animals was taken as an evidence for a prominent role of TGF-β

in T_{reg} cell maintenance [49]. We, however, observed upon removal of TR2 from T_{reg} cells an increase in their population size that went along with their increased proliferation. In the CD4-Cre/TR2$^{f/f}$ model T_{reg} cells and their thymic precursors mice also showed a hyperproliferative phenotype [49] but in the periphery exaggerated Bim-dependent apoptosis resulted in the drastic reduction of T_{reg} cell numbers [108]. Interestingly, the thymic T_{reg} cell expansion seen in TR1-deficient thymocytes of 1 week old mice was dependent on IL-2 production by mutant conventional CD4$^+$ thymocytes [109], but also these cells fail to survive in the periphery. In the periphery of a DN-TR2 model the CD25$^+$ cells were expanded as well, but without analysis of FoxP3 expression it remains unclear whether these cells were T_{reg} or activated conventional T cells [110]. Furthermore, it cannot be excluded that the seemingly similar observation to our model is the result of the modified instead of absent signalling, because transgene expression was also initiated at the DP thymic stage [49-50]. In further experiments we have investigated whether mature T_{reg} cells were still retaining their characteristics after removal of TR2. In contrast to data from a constitutive model [107], we did not see a reduction of Foxp3 levels in peripheral CD25$^+$ cells in the absence of TR2. Also, expression of the T_{reg} surface markers CTLA4 and GITR was unchanged, in contrast to observations in young CD4-Cre/TR2$^{f/f}$ mice (data not shown). Furthermore, we did not observe any decrease in suppressive abilities of T_{reg} cells lacking TR2, both *in vitro* and *in vivo*. We excluded that a defect on TR2-deficient effector T cells precluded T_{reg} cells from exerting their suppressive activity, in contrast to effector T cells expressing a dominant negative mutant of TR2 which were shown to be resistant to suppression by wt T_{reg} cells in colitis, diabetes, or tumor models [94, 233-235]. In contrast to our findings, a DN-TR2 transgene showed deficient suppressive abilities within its T_{reg} cell compartment [94, 107]. This result may be the consequence of modified but not completely absent TR signal due to the dominant-negative receptor. Taken together, ablation of TGF-β signalling already during thymic development seems to lead to intrathymic hyperproliferation of T_{reg} cells which cannot

survive in the periphery. In contrast, when TR2 is removed from already established peripheral T_{reg} cells, these cells keep their regulatory characteristics and undergo increased proliferation, thus resulting in an increased T_{reg} population size.

Therefore, from our observations and in consistency with other experimental data, we propose that TGF-β functions as a control factor keeping T_{efm} and T_{reg} populations at bay. Because it was shown that TGF-β can control its own expression by an autoregulatory mechanism [236], we investigated TGF-β1 expression in presence and absence of TR2 in the different $CD4^+$ T cell sub-populations. Yet, since we did not find TGF-β1 production to be specifically restricted to these two populations, we can rule out that a direct feedback mechanism for control of population sizes is at play. The control may thus include other cell types, such as dendritic cells, which were described to be critical for suppression of autoimmunity by activating matrix-bound TGF-β [237].

The role of TGF-β during differentiation of $CD4^+$ T cells into the Th1 and Th2 lineages

TGF-β was shown to be a potent inhibitor of Th1 and Th2 differentiation *in vitro*. Addition of TGF-β to predisposing conditions for Th1 and Th2 subset resulted in down-regulation of expression of the defining transcription factors T-bet and GATA-3, respectively [197-199, 238-239]. In accordance, studies of mice constitutively lacking TGF-β signalling in T cells showed that *ex vivo* restimulation of TR2-deficient T cells with anti-CD3 and anti-CD28 antibodies for 24 hours led to an increased IFNγ production [49, 197]. Enhanced Th1 differentiation was also described *in vivo* in the T-cell specific gene-deficiencies for TGF-β1 and its signalling pathways [49, 95]. These effector Th1 cells were able to infiltrate organs and establish a highly aggressive self-reactive T cell-mediated autoimmunity. In a recent report, the inhibitory role of TGF-β for Th1 differentiation was further assessed, using mice

with a T cell-specific deficiency for the down-stream targets of TGF-β signalling Smad2 and Smad3. It was shown that TGF-β lost its ability to inhibit differentiation of these T cells into the Th1 subset [202].

However, in our skewing experiments in Th1 predisposing conditions we could not detect any differences in polarization capacities of TR2-deficient T cells to differentiate into Th1 subset compared to control T cells. Neither IFNγ expression nor T-bet expression were upregulated as measured by intracellular staining and ELISA. After culture the cells isolated from tamiCD4TR2 cells in Th2 polarization we did not observe differences in T cell numbers of tamiCD4TR2 or control TR2 lymphocytes expressing GATA-3 and IL-4. In the supernatants of the cultures though, significantly higher amounts of IL-5 were produced by tamiCD4TR2 cells than by control T cells. The different observations of IL-4 and IL-5 production could be due to a differential regulation of IL-4 and IL-5 expression by GATA-3. A direct involvement of GATA-3 in IL-5 promoter activation has been demonstrated but it seems that IL-4 gene expression needs multiple factors such as c-Maf or NF-IL6, which probably act in concert with GATA-3 to induce gene expression [173-174, 240].

It has also been reported before that TGF-β has different effects on naïve and effector T cells *in vitro* [241-242]. One study showed that TGF-β could inhibit cytokine production of memory Th1 but not Th2 cells [243]. By use of our model, mature $CD4^+$ T cell subpopulations could be sorted before the deletion of TR2 and cultured separately under skewing conditions. This might allow an even more detailed analysis of the TGF-β signalling on priming or recall responses of T cells.

In contrast to previously published reports we did not observed after *in vivo* ablation of TR2 in $CD4^+$ T cells that they spontaneously differentiate into Th1 or Th2 cells [49, 110]. Also, the production of IFNγ was only slightly upregulated ex vivo and the expression of Th2 cytokines

was hardly detectable. Upon CD3/28 stimulation of un-immunized T cells also no difference in Th1 and Th2 cytokines could be observed as well.

In addition to the steady-state analysis, we investigated the immune response in tamiCD4TR2 mice and Th differentiation after immunization with the model antigen KLH in CFA. This immunization was reported to bias the overall immune response towards Th1 with increased IL-2 and IFNγ, but not IL-4 production [244]. In accordance we detected high amounts of IFNγ in culture supernatants of restimulated KLH-primed cells. According to reports that established a role for TGF-β in blocking Th1 responses [49-50, 95, 110, 197-199, 238-239], we would have expected an enhanced differentiation into the Th1 subset along with increased IFNγ and IL-2 production after immunization. The produced amounts of IFNγ and IL-2 did not differ between TR2-deficient and control cells after 48 or 72 h. However, by ELISPOT assay we observed significantly more IFNγ-producing cells among tamiCD4TR2 T cells, which suggested an increased differentiation of TR2-deficient into Th1 subset upon KLH immunization *in vivo*. This however would implicate that cytokine production per cell was reduced. In contrast to the observations made by *in vitro* skewing, TR2-deficient T cells produced significantly less of IL-4 in recall response. IL-10 production was also significantly reduced among TR2-deficient T cells. The expression of immunoregulatory IL-10 by T cells has also been shown to be dependent on TGF-β signalling [245-248].

The observation of reduced Th2 cytokine production in restimulated TR2-deficient T cells contrasts the reported role of TGF-β inhibiting Th2 differentiation and the described *in vitro* findings. To exclude mainly Th1 responses in the used *in vivo* setup, KLH immunization with a Th2 response-promoting adjuvant like Ribi [244] or Alum [249] could be performed.

An increased number of T_{reg} cells in tamiCD4TR2 mice could potentially inhibit $CD4^+$ T cell differentiation into Th1 lineage. They might have a compensatory effect on differentiation of T cells by exerting their suppressive functions independent of TGF-β. By generation of mixed

bone marrow chimeras we wanted to dilute regulatory T cells which strongly proliferated upon loss of TR2. After 5 weeks post reconstitution the CD45.2$^+$ population expanded to the same extend as wt CD45.1$^+$. Thus at the end we were not able to conclude if the lower expression of the cytokines by tamiCD4TR2 CD4$^+$ T cells in the chimeric mice was the caused by enlarged population of T_{reg} cells or was direct effect of missing TGF−β signalling. In addition we observed that the wt CD4$^+$ T cells in experimental mixed bone marrow chimeric mice produced more INFγ and IL-17 that CD4$^+$ T cells in control chimeras.This could suggest that compensatory mechanisms exist that keeps within limits the level of certain cytokines. Further experiment performed with mixed bone marrow chimeras would have to be performed to investigate the expression of cytokines upon removal of TR2 but the transgenic population would have to be diluted with wt cells at least 1:10.

In the skewing conditions towards Th1 and Th2 phenotype and after CD3 and CD28 stimulation we also analysed the expression of another cytokine that are important for effector function of T cells: GM-CSF [250]. Analysis performed on day three of culture showed significantly more GM-CSF producers among TR2-deficient T cells than among control TR2 cells even though Th1 skewing conditions rather inhibit the production of GM-CSF. Thus, the absence of TR2 in CD4$^+$ T cells promoted GM-CSF production. Similar results were obtained ex vivo after analysis of tamiCD4TR2 mice without immunization. In contrast, *in vivo* primed TR2-deficient T cells produced less GM-CSF upon recall.

In conclusion, we did not observe in our model that peripheral CD4$^+$ T cell after abrogation of TR2 signalling differentiate spontaneously into Th1 or Th2 lineage and even after immunization the difference in cytokine production are minor.

The role of TGF-β during differentiation of CD4+ T cells in to the Th17 lineage

After the description of a new IL-17 producing T helper subset (Th17), distinct from Th1 and Th2 subset [181-182, 251], several studies showed that TGF-β signalling was essential for terminal differentiation of cells into this lineage [95, 142, 185-187]. We examined the role of TGF-β for Th17 cells differentiation in our model *in vitro* and *in vivo*. For *in vitro* skewing we used culture conditions that included TGF-β as an essential cytokine to predispose naïve T cells to differentiate into Th17 subset. In accordance, only a little fraction of TR2-deficient T cells differentiated towards the Th17 phenotype. The percentage of $CD4^+$ T cells that expressed both, RORγt and IL-17 was reduced to about 10 % of control $CD4^+$ T cells. Surface staining of TR2 revealed that approximately 10-15 % of T cells retain the receptor after tamoxifen-treatment. Thus, these TR2-proficient T cells from tamiCD4TR2 animals could account for the small fraction polarizing into IL-17 producers. Still, so far we cannot exclude that also TR2-deficient cells might have differentiated towards IL-17 producing phenotype. In Th17 skewing condition a higher percentage among TR2-deficient T cells expressed IFNγ than among control T cells. This suggests that $CD4^+$ T cells which could not differentiate into the Th17 subset, polarized into the Th1 phenotype, in line with results found in a recent study [252].

In vitro studies have shown that an antibody cocktail inhibiting Th1 and Th2 differentiation promoted polarization of Th17 subset [187]. This suggested that TGF-β functions in Th17 differentiation by inhibiting the differentiation of Th1 and Th2 subset. Though, in the same and other studies [185-186] a more active role of TGF-β was revealed as neutralizing TGF-β antibodies abrogated Th17 differentiation completely. Also Smad2 and 3, which are regulated by TGF-β signalling were shown to be essential for the development of fully functional Th17 cells [202]. To further assess the role of TGF-β for Th17 polarization, it might be possible to establish other *in vitro* conditions for Th17 differentiation. Very recently, it was shown that

TGF-β could be substituted by IL-1β to induce differentiation of Th17 cells *in vitro* [253]. The strong reduction of IL-17 production was also observed after CD3/CD28 stimulation of not primed CD4 T cells from tamiCD4TR2 mouse.

We investigated also the production of IL-17 in vivo. After adoptive co-transfer of tamiCD4T2 cells together with wt cells into lymphopenic host, the IL-17 expression by the transgenic population was strongly reduced in the spleen and mesenteric lymph nodes. Similar results were obtained after immunization mixed bone marrow chimeras with KLH. In experimental chimeras TR2-deficient T cells expressing RORγt and IL-17 were strongly reduced compared to the wild type T cell population. As described for IFNγ expressing cells, this might suggest a compensatory effect, which would be in accordance with published data that TGF-β signalling is essential for Th17 differentiation.

TGF-β signalling during experimental autoimmune encephalomyelitis

The studies, in which the role of TGF-β in EAE was investigated, showed that the action of this cytokine is context-dependent and site-specific. On the one hand, administration of TGF-β ameliorated disease [133-135] and was shown to have profound effects on remission of sick animals [9]. TGF-β signalling in innate immune cells was shown to be protective [139] while on the other hand, loss of TGF-β signalling in T cells led to a reduced EAE susceptibility [95, 142]. Production of TGF-β by glial cells was reported to promote CNS infiltration of pathogenic T cells [254-255]. By use of our tamoxifen-inducible model we thus wanted to investigate the role of TGF-β signalling in mature $CD4^+$ T cells during EAE. This question was previously approached by use of models for T cell-specific abrogation of TGF-β signalling. In the CD4-dnTGF-βRII model, animals were less susceptible to EAE which was only connected by the authors with a reduced Th17 development [187]. In accordance, local administration of neutralizing TGF-β antibodies inhibited disease development in wild type animals, which was proposed to prevent Th17 differentiation. T cell-produced TGF-β also

seemed to be essential for the development of encephalitogenic Th17 cells [95]. Animals that lacked T cell-produced TGF-β showed only mild clinical symptoms. However, expansion of primed Th17 cells in presence of TGF-β reduced their encephalitogenicity in an adoptive transfer EAE model [143].

Ours experiments with tamiCD4TR2 mice revealed that animals which had been actively immunized with MOG_{35-55} peptide were as susceptible to EAE as wild type animals. Because tamoxifen treatment showed a considerable side-effect and reduced disease severity, a new setup was established in order to elucidate the role of TGF-β signalling on development of the disease. Cells from tamiCD4TR2 2d2 mice were transferred into $RAG1^{-/-}$ animals followed by active immunization with MOG peptide. All recipients of 2d2 tamiCD4TR2 and 2d2 control TR2 cells developed a severe terminal disease. No differences between recipients of 2d2 tamiCD4TR2 cells and recipients of 2d2 control TR2 cells was in the course of the disease. Thus, TR2-deficient 2d2 T cells were able to establish EAE to a similar degree and incidence as wild type 2d2 cells. However, as the severity of disease in this model increased very fast, this strong outcome may have masked minor differences in disease course upon ablation of TR2. Additionally in 2d2 TCR transgenic mice the population of regulatory T cells is reduced which make it even more difficult to elucidate their role in EAE after abrogation of TGF-β signalling. To better investigate the role of TGF-β signalling during EAE different experimental setups will have to be tested in which tamoxifen treatment did not interfere with immunizations and disease course is milder.

General implications of results in the context of technology and knowledge

Taken together, our study suggests that several misconceptions about TGF-β function in peripheral lymphatic organs are the result of gene ablation during T cell development. By restricting the genetic defect (TR2-deficiency) to mature T cells we could show clearly that

TGF-β signalling is not essential for the suppression of autoimmunity and the maintenance of a functional T_{reg} cell pool. Instead it is required for inhibition of overt proliferative activity of T_{reg} and T_{efm} cells. In addition, we could not observe any role in IL-2 signalling and IL-2 production. Furthermore tami$CD4^+$ T cells did not acquire spontaneously a Th1 phenotype described before. Thus, TGF-β1 remains a cytokine with critical function in the regulation of T cells, yet its role in peripheral tolerance seems to have been somewhat overinterpreted in the past.

Our new tamoxifen-inducible CD4-$CreER^{t2}$ strain is the first mouse model which allows efficiently inducing or depleting the gene of interest solely in $CD4^+$ T cells. Thus this transgenic mouse can be a very useful tool in many immunological projects.

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ABBREVIATIONS

APC	Antigen presenting cell
CD	Cluster of differentiation
CNS	Central nervous system
cTEC	Cortical thymic epithelial cell
DN	Double negative thymocyte
DP	Double positive thymocyte
DC	Dendritic cell
EAE	Experimental autoimmune encephalomyelitis
IBD	Inflammatory bowel disease
iCD4TR2	CD4-CreER^{t2}/TGFbRII $^{f/f}$
KLH	Keyhole limpet hemocyanin
LN	Lymph node
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
p.a.	Post application
PMA	Phorbol 12-myristate 13-acetate
SLE	Systemic lupus erythematosus
SP	Single positive thymocyte
tamiCD4TR2	Tamoxifen-treated CD4-CreER^{t2}/TGFbRII $^{f/f}$
TCR	T cell receptor
Th	T helper
T_{efm}	Effector memory T cells
T_n	naïve T ce
T_{reg}	Regulatory T cells
nT_{reg}	Natural regulatory T cells
TR1	TGF-βRI, transforming growth factor β receptor I
TR2	TGF-βRI, transforming growth factor β receptor II
wt	Wild-type

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